CHARACTERIZATION OF BIOAEROSOLS FROM CAGE-HOUSED AND FLOOR-HOUSED POULTRY OPERATIONS

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ABSTRACT

Background. Dust and endotoxin levels are higher in bioaerosols from floor-housed (FH) poultry operations than cage-housed (CH) poultry facilities. Workers from CH operations have reported a greater prevalence of respiratory symptoms than FH workers. The negative respiratory symptoms observed in workers are typically attributed to endotoxin. However, other components of poultry bioaerosols and their effects on human health, such as bacteria, antibiotics and archaea, are poorly understood. Bacteria have been detected in intestinal, fecal, litter, and air samples from poultry operations. Chicken fecal bacteria differ depending on bird age and antibiotic use, which differ between CH and FH facilities. Antibiotics are used in CH and FH poultry operations to lower the likeliness of disease transmission. In FH facilities, antibiotics may also be used at sub-therapeutic levels for growth promotion. Low levels of antibiotic create a selective pressure towards antimicrobial resistance (AMR) in chicken fecal bacteria. Archaea have been detected in ceca, fecal, litter and house fly samples from poultry facilities but have not been investigated in bioaerosols. However, archaea have been detected in swine and dairy bioaerosols and can induce airway inflammation. Further understanding of poultry bioaerosols, with a comparison of those from CH and FH operations, will aid in the development of management practices to reduce worker exposure and response. *Objective.* The objective of these studies was to compare bioaerosols from CH and FH poultry facilities. Specifically, levels of dust, endotoxin, total bacteria, bacterial species, antimicrobial resistance genes and methanogenic archaea were examined.

Methods. Bioaerosols were collected from fifteen CH and fifteen FH poultry operations using stationary area samplers as well as personal sampling devices. Dust was measured by gravimetric analyses. Limulus Amoebocyte Lysate (LAL) assays were used to quantify endotoxin. Bacteria and archaea concentrations were measured by quantitative PCR. Bacterial and archaeal diversity was investigated using PCR followed by denaturing gradient gel electrophoresis (DGGE) and sequencing. AMR genes were detected using end-point PCR. *Results.* Dust ($p<0.001$), endotoxin ($p<0.05$), total bacteria ($p<0.05$), *Enterococcus* ($p<0.001$), *E. coli* ($p<0.001$) and *Staphylococcus* ($p<0.001$) were more concentrated in bioaerosols from FH poultry operations than CH bioaerosols. Methanogenic archaea ($p<0.001$) and *C. perfringens* ($p<0.05$) were significantly higher in bioaerosols from CH facilities than FH bioaerosols. Zinc bacitracin resistance gene (*bcr*R*)*, erythromycin resistance gene (*erm*A*)*, and tetracycline resistance gene (*tet*A/C*)*, were more prevalent in bioaerosols from FH facilities than CH bioaerosols ($p<0.01$, $p<0.01$ and $p<0.05$, respectively). *Conclusions.* Bioaerosols from CH and FH poultry operations are significantly different, suggesting that CH and FH workers are exposed to significantly different environments. Bacterial diversity, *C. perfringens*, archaea, and/or unmeasured components of bioaerosols may be contributing to the greater prevalence of respiratory symptoms observed in CH workers. Each barn type may require specific remediation methods. *Future directions*. In order to better understand the role of bioaerosols in poultry worker respiratory dysfunction, it will be necessary to examine airway inflammation following exposure to bioaerosols, or components of bioaerosols, from each poultry barn type.

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I dedicate this work to my family. To my father Jerald—who taught me to believe that I can accomplish anything. To Shelley—who is an incredible mentor and example of the kind of woman I want to be. To my brother Jeremy—who has given a lifetime of friendship. To my sister Jocelyn—who is always a source of light. To my Keli—who shares my soul. And to my son Orison—who has brought true meaning to my life.

In memory of my mother Merrilee—who was a courageous, beautiful woman.

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LIST OF ABBREVIATIONS

A ..... area

AMR ..... antimicrobial resistance

ARG ..... antimicrobial resistance gene

Asp ..... aspartate

BAL ..... bronchoalveolar lavage

BLASTn ..... basic local alignment search tool nucleotide

bcr ..... bacitracin

C ..... Celsius

CFU ..... colony forming unit

CH ..... cage-housed

CHUL ..... Centre Hospitalier de l'Université Laval

CO_2 ..... carbon dioxide

CpG ..... cystosine-phosphate-guanine

DGGE ..... denaturing gradient gel electrophoresis

DNA ..... deoxyribonucleic acid

erm ..... erythromycin resistance methylase

EU ..... endotoxin unit

EUB ..... eubacteria

FAM 6-carboxyfluorescein

FEF_{25-75} ..... forced expiratory flow rate between 25% and 75% FVC

FEV_1 ..... forced expiratory volume in 1 second

FH ..... floor-housed

FVC ..... forced vital capacity

GAPDH ..... glyceraldehyde-3-phosphate dehydrogenase

GC ..... guanine-cytosine

Gly ..... glycine

H_2 ..... hydrogen

HDE ..... hog dust extract

$IB^{TM}FQ$ ..... Iowa Black Fret Quencher

ICAM ..... intracellular adhesion molecule

IFN ..... interferon

IL ..... interleukin

Ile ..... isoleucine

LAL ..... limulus amoebocyte lysate

LPS ..... lipopolysaccharide

LTA ..... lipoteichoic acid

m^3 ..... cubic metres

Mal MyD88-adaptor-like

MAPK ..... mitogen-activated protein kinase

mg ..... milligram

mRNA ..... messenger ribonucleic acid

MRSA ..... methicillin-resistant *Staphylococcus aureus*

MUC ..... mucin

MyD ..... myeloid differentiation factor

NF-κB ..... nuclear factor kappa B

ng.....nanogram

ODTS.....organic dust toxic syndrome

P.....personal

PAMP.....pathogen-associated molecular pattern

PBS.....phosphate buffered saline

PCR.....polymerase chain reaction

PDE.....poultry dust extract

PG.....peptidoglycan

PI3K.....phosphatidylinositol 3-kinase

ppm.....parts per million

PVC.....polyvinyl chloride

qPCR.....quantitative PCR

qRT-PCR.....quantitative reverse transcriptase-PCR

rDNA.....ribosomal deoxyribonucleic acid

rRNA.....ribosomal ribonucleic acid

sp......species (singular)

spp......species (plural)

TAE.....tris-acetate-EDTA

tet.....tetracycline

Thr.....threonine

TIR.....toll/interleukin-1 receptor

TLR.....toll-like receptor

TNF.....tumour necrosis factor

TRAM.....TRIF-related adaptor molecule

TRIF.....TIR domain-containing adaptor inducing IFN-beta

tuf ..... Tu elongation factor

UDG ..... uracil N-glycosylase

µg ..... microgram

µm ..... micrometre

UPGMA ..... unweighted pair-group method using arithmetic averages

1.0 INTRODUCTION

1.1 Literature review

1.1.1 Poultry barn environments

Cage-housed and floor-housed operations are two common types of poultry housing facilities. In cage-housed operations birds are housed in cages for egg production and in floor-housed operations birds are housed on the floor for meat production. There are a number of differences in the two types of poultry operations including time spent by the workers in direct contact with birds, predominance of female poultry in cage-housed facilities, age of birds, length of time birds spend in housing and housing management practices. Previous data show that personal total dust exposures are significantly higher in floor-housed versus cage-housed operations [1, 2]. However, a trend towards higher endotoxin load (EU/mg) in cage barns was observed [1]. Significant differences in respiratory symptoms are observed between cage-housed and floor-housed workers. Current and chronic phlegm occurred more frequently in workers from cage-housed facilities and endotoxin load was shown to be a significant predictor of chronic phlegm [1]. Very few studies differentiate between these two types of poultry housing despite the known differences in management practices and worker response. It is possible that the type of housing may influence levels of environmental contaminants in the dust.

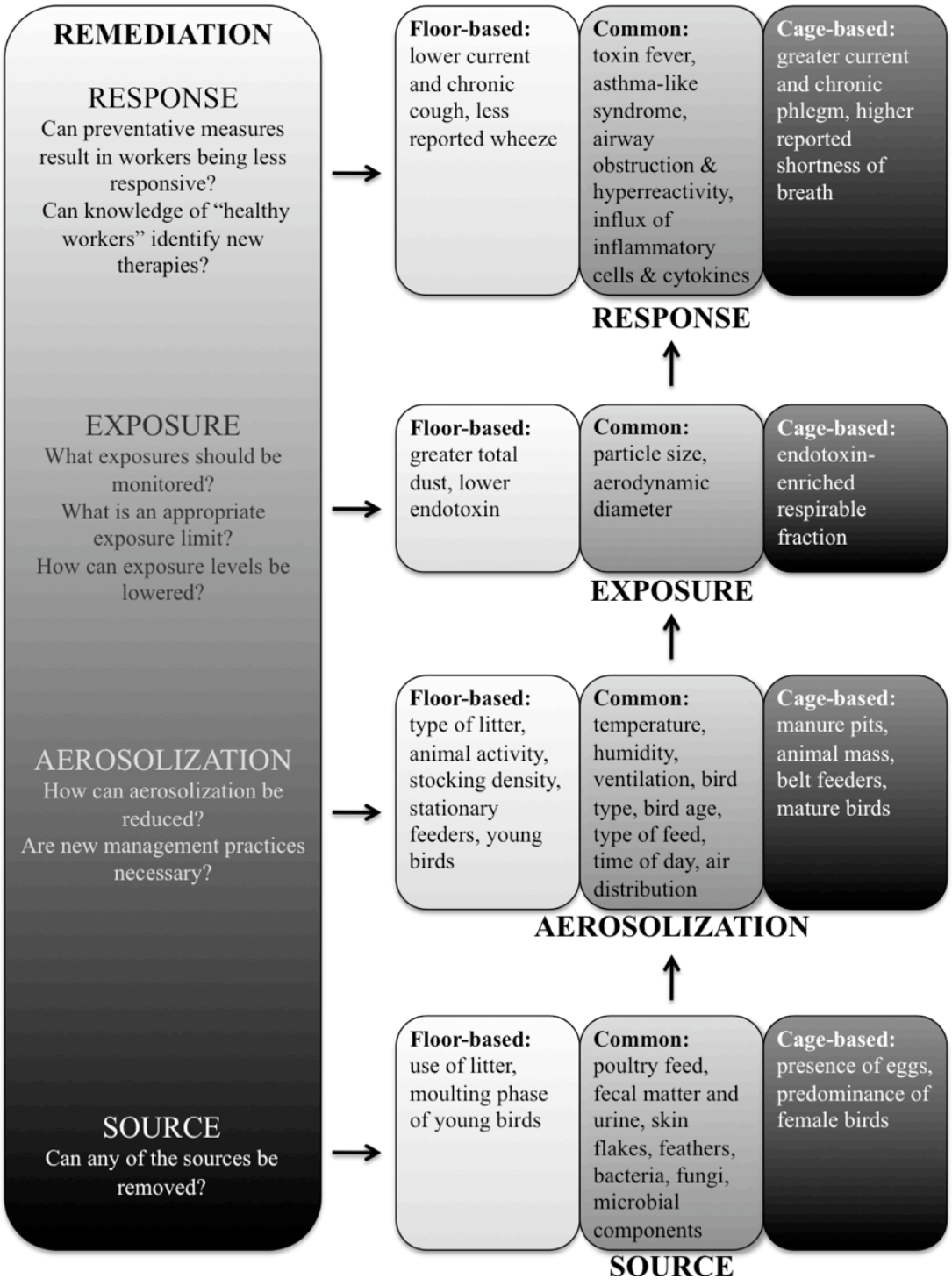

Figure 1. Aerobiological pathway of dust in poultry facilities. Common factors influencing each stage of the pathway are indicated in the grey boxes, specific cage-housed factors are highlighted in black boxes and floor-housed factors are outlined in white boxes. Remediation opportunities for each stage of the pathway are indicated at the left. Figure 1 has been published previously [3]. It is reproduced here with permission of the copyright owner (BioMed Central).

1.1.2 Aerobiological pathway

The aerobiological pathway that results in bioaerosol production includes the source, aerosolization and dispersal, exposure, response and remediation (Figure 1) [3]. Further understanding of the source and aerosolization of dust in poultry operations will aid in the development of management practices to reduce worker exposure and response. Examination of the two types of poultry operations separately may reveal different means of improving respiratory health in the two types of workers.

1.1.2.1 Sources

Dust is a complex mixture of organic and inorganic particles, different gases, and aerosol droplets. Organic dust components can be further divided into non-viable and viable particulate matter, or bioaerosols [4]. The sources of organic dust from a poultry facility include dried fecal matter and urine, skin flakes, ammonia, carbon dioxide, pollens, feed and litter particles, feathers (which produce allergen dandruff), grain mites, fungi, spores, bacteria, viruses and their constituents, peptidoglycan, β-glucan, mycotoxin and endotoxin [1, 4-7]. Down feathers and crystalline dust are the major physical components of dust. Crystalline dust originates from urine [6]. The solid components of dust act as a transport vector for noxious gases and biological contaminants, allowing these to be inhaled into the lungs [8].

Characterization of dust sources is important in order to identify those sources that may, or may not, be removed (Figure 1) [3]. For example, endotoxin originates from Gram-negative bacteria found in fecal matter, urine, litter and feed particles. Although the presence of feces, urine, litter and feed are all intrinsic to poultry production, the

types of feed and litter may alter the types and levels of bacteria, providing a potential means for lowering sources of endotoxin.

1.1.2.1.1 Particle size

Dust particles vary in size and shape in animal confinement buildings [8]. Differentiation between particle size fractions is important in health studies in order to estimate penetration of dust within the respiratory system. Particles greater than 10μm may be deposited in the nasopharyngeal region by impaction [9]. Impaction depends on air velocity and particle mass. Particles with an aerodynamic diameter between 0.5μm and 5μm can deposit in the tracheobronchial region by sedimentation [9]. Sedimentation occurs when air velocity decreases and particles settle. Particles less than 0.5μm may be deposited in the alveolar region by diffusion [9]. Diffusion occurs due to random particle movement. Particles between 0.4μm and 0.7μm are too large for diffusion and too light for sedimentation and thus, have minimal deposition [10]. The particle size range with the largest percentage of deposition in the lungs is 1-2μm in aerodynamic diameter [7].

Approximately 50% of particles less than 5μm aerodynamic diameter entering the respiratory system will reach the alveoli. Therefore, particles greater than 5μm are inhalable, while particles 0.5-5μm are respirable [7, 11]. Respirable dust accounts for ~18% of total dust mass [7].

1.1.2.1.2 Ammonia

Ammonia is a colourless, pungent gas produced by the decomposition of uric acid in manure by microbes. Effects on human health related to exposure to ammonia are

nausea, vomiting, chest pain, difficulty breathing, headaches, and impaired mucus flow and ciliary action in the trachea, causing increased susceptibility to bacterial infection [12]. The pH, temperature and moisture levels of litter have an impact on ammonia levels in floor-based operations [5, 7]. New litter typically has a pH of 5-6.5. *Bacillus pasteurii*, one of the uricolytic bacteria, requires a pH of 8.5 to grow. Therefore, ammonia production from new litter will be slow at first but eventually the pH will rise, facilitating ammonia production. At higher temperatures, not only is bacterial activity and ammonia production increased but gas transfer from litter to air is also increased. Low moisture reduces microbial activity and ammonia production, whereas very high moisture also reduces ammonia production due to anaerobic conditions [7]. In cage-based operations the ammonia concentration depends on manure storage and removal systems as well as ventilation rate and airflow patterns [5]. The use of gas heaters in poorly insulated houses can increase condensation levels, causing wet litter and higher ammonia levels [7].

Some of the respiratory symptoms observed in poultry workers are a result of exposure to ammonia and other volatile organic compounds. The solubility of ammonia with water may cause it to be trapped in the upper airways but ammonia can travel deeper into the lungs when carried on dust particles. Thus, ammonia and dust exposure have been shown to cause synergistic health effects [6].

1.1.2.1.3 Endotoxin

Endotoxin is the most frequently reported environmental contaminant in poultry dust. Endotoxin is the family of lipopolysaccharide (LPS) fragments that coat the outer

membrane of Gram-negative bacteria [13]. LPS is composed of three structural elements: a core oligosaccharide, an O-specific chain made up of repeating sequences of polysaccharides and a lipid A component, which is responsible for the toxic effects of LPS exposure [14]. Common occupational sources of exposure include livestock, grain dust, and textiles, but significant concentrations also occur in the household from pets, carpeting and indoor ventilation systems. Endotoxin has also been found in tobacco smoke and particulate matter in air pollution [13]. In poultry operations, endotoxin originates from Gram-negative bacteria that can be found in fecal matter, urine, litter, grain and other vegetable matter in poultry feed [7, 15, 16]. Endotoxin can be measured by the Limulus Amoebocyte Lysate-based (LAL) bioassay, which measures biological activity of endotoxin, or by mass spectrometry, which can quantify endotoxin biochemically through detection of LPS-characteristic 3-hydroxy fatty acids [17].

1.1.2.1.4 Bacteria and fungi

Microorganisms in airborne particles are often associated with the negative health effects associated with the poultry industry [8]. Microorganisms exist suspended freely in the air as well as attached to dust particles [7]. Bacteria are generally 1-2μm in diameter and associate with dust particles that are in the respirable size range [5]. The aerobic bacteria common in poultry facilities include: *Bacillus* spp., *Micrococcus* spp., *Proteus* spp., *Pseudomonas* spp., *Staphylococcus* spp. and *E. coli* and common anaerobic bacteria are *Clostridia* spp. [18]. Measures in experimental poultry houses showed that 80% of airborne bacteria were Gram-positive aerobes and only 7-17% were Gram-negative rods when litter was present [7]. Gram-positive and Gram-negative bacteria

contain different pathogen-associated molecular patterns (PAMPs). LPS is a specific component of the Gram-negative bacterial cell wall, while peptidoglycan (PG) and lipoteichoic acids (LTA) are major cell wall components of Gram-positive bacteria [19, 20]. Unmethylated CpG DNA is abundantly found in microbial DNA [20].

Airborne fungi present in poultry facilities include *Cladosporium* spp., *Aspergillus* spp., *Penicillium* spp. and less commonly, *Alternaria* spp., *Fusarium* spp., *Geotrichum* spp. and *Streptomyces* spp. [18, 21].

Management processes that control relative humidity, temperature, type and age of the litter can influence the types and levels of fungi and bacteria present in poultry facilities [7]. In floor-housed operations it has been shown that levels of airborne dust, endotoxin and bacteria increase throughout the growth cycle of the chickens [4]. This increase parallels the increase of biomass (number of birds x bird weight) during the growth cycle and corresponding higher levels of skin debris and feathers.

1.1.2.1.5 Archaea

Archaea are similar to bacteria in size and shape [22], have Bacteria-like metabolism, and no nucleus or organelles [23]. However, Archaea are similar to Eukaryotes in their genetic, transcriptional and translational pathways [22, 23]. Archaea were named as such because they were originally detected in extreme environments, the most primitive locations on earth [22]. However, they are also found in non-extreme environments, digestive tracts of many animals [23, 24], and intestinal, vaginal and oral mucosa in humans [22]. Gastrointestinal archaea use various organic substrates, including alcohols, organic acids, CO_2 and H_2, to produce methane [25].

Methanogenic archaea have been detected in broiler fecal samples [26] and ceca of layer hens [27]. Airborne archaea have been detected in bioaerosols from dairy and swine barns at concentrations of 10^4 to 10^6 archaea/m^3 and 10^6 to 10^8 archaea/m^3, respectively [28, 29]. Archaea have never been examined in poultry bioaerosols. The airway response following exposure to airborne archaea is poorly understood. Recent results show that two methanogens, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, induce airway inflammatory responses following intranasal exposure in mice [30]. Therefore, it is possible that airborne archaea may play a role in poultry worker respiratory health outcomes.

1.1.2.1.6 Antimicrobial resistance

Antimicrobials include antibiotics and disinfectants that eliminate and/or inhibit the growth of microorganisms, including bacteria. Antimicrobial resistance is the ability of a microorganism to survive and/or grow in the presence of antimicrobials. A bacterial strain is defined as antibiotic resistant when a genetic alteration allows it to tolerate an antibiotic concentration much higher than the concentration that inhibits development of most strains of the same bacterial species [31]. This ability is due to the acquisition of antimicrobial resistance genes, which occurs through genetic mutation or transmission between bacteria [32]. It has been observed that the likelihood of antimicrobial resistance increases the longer an antibiotic is used. Although scientists do not fully understand how antimicrobial resistance originates, they believe antimicrobial use in animals is a major contributing factor. The use of antibiotics in food production is associated with the development of antibiotic-resistant bacteria in these animals [33]. In many countries,

over 50% of antibiotics produced or imported are administered to animals [32]. Most of the antibiotics used in food production are also used in human medicine. In humans, antimicrobial resistance results in heightened financial costs due to extended patient treatment, support of patients with chronic resistant infection, maintaining antimicrobial resistance surveillance programs and the development of new treatments. To date, the societal response to antimicrobial resistance has been to develop new drugs. However, it is predicted that the future supply of new, safe, effective and affordable antimicrobials will diminish. Hence, a more sustainable approach to food production that reduces antimicrobial resistance health issues needs to be developed.

Antibiotics are used in food production animals to control or prevent infection (prophylaxis), for growth promotion and to treat disease (therapy). Prophylaxis is used during periods of high-risk for disease. Growth promoters are antimicrobials used at low doses in feed or water to stimulate animal growth, increasing daily weight gain and/or feed efficiency. Although the benefits of therapeutic antimicrobial use are high, the benefits of their use as growth promoters are in question. Growth promoters have been shown to increase feed efficiency and weight gain (1-15%), which is low on the per-animal basis but may be large on a industry-wide basis [32]. However, an increase of only 1% in cost of production is hypothesized for producers that do not use growth promoters [34]. The benefits of growth promoters are generally greater in poor hygienic conditions, suggesting that management practices (vaccines, biosecurity) could reduce the need for antimicrobials.

Many antibiotics are registered as growth promoters for food production in Canada, including: tylosin, penicillin, tetracycline, erythromycin, sulfamethazine,

bacitracin and virginiamycin [32, 35, 36]. In the poultry industry, isolates of *Campylobacter* spp. have shown resistance to erythromycin and tetracycline [35]. *Enterococcus* spp. strains display multi-antimicrobial resistance [36-38]. *E. coli* and *Salmonella* spp. strains show resistance to gentamicin, neomycin and tetracycline [35]. *Staphylococcus aureus* strains display resistance to penicillin and tetracycline [32].

Some of the antimicrobials used in poultry production are similar or identical in chemical structure to antimicrobials used to treat human infections [39]. Some of the bacteria found in poultry operations are human pathogens and antimicrobial-resistant bacteria can be transferred to humans, which is a health concern. For example, fluoroquinolone-resistant *Campylobacter* in poultry operations is transferred to humans and can cause infections [39]. Antimicrobial resistance can result in adverse human health effects through direct transfer of resistance genes from zoonotic infections or the indirect transfer of resistance genes from commensal animal bacteria to human pathogens. For example, commensal *E. coli* is often a reservoir of antimicrobial resistance genes although it is rarely the cause of disease. These genes can then be transferred to pathogens of the gut, such as *Salmonella* spp. [40]. Transmission of pathogens can occur through direct contact, contaminated water, environment and food [41]. The consumption of retail meat has been shown to be associated with antimicrobial resistant bacterial infections [42]. Respiratory transmission of pathogens and antimicrobial resistance genes to agricultural workers is poorly characterized in the literature. However, airborne horizontal transmission of pathogens among animals has been reported in the poultry and swine industries [43, 44]. Airborne bacteria are present at higher concentrations downwind from food production facilities [45] and these bacteria

are shown to be higher in antimicrobial resistance than bacteria found upwind of food production operations [46]. Multidrug resistant bacteria have been recovered from inside and up to 150m downwind from swine confinement buildings [47]. There is an increased incidence of respiratory illness in communities downwind from food production facilities [45].

Antibiotic-treated feed particles are a source of organic dust in food production facilities and antibiotics have been isolated in settled dust [48]. Antibiotics have been isolated from inhalable and respirable dust sizes [49]. Therefore, workers and producers have a consistent occupational exposure to antibiotics, promoting antimicrobial resistant respiratory or intestinal infections [41]. In fact, the number of hours spent in a food production facility is associated with antimicrobial resistance [41]. It is becoming more evident that workers, producers, farm residents and neighbouring communities are potential reservoirs of multiple antimicrobial resistance genes.

1.1.2.2 Aerosolization

The contaminants described in the preceding sections can be readily aerosolized and dispersed throughout the poultry barn environment. Aerial dust concentrations are affected by the rate of aerosolization, settling velocities and resuspension rates of airborne particles [8]. Therefore, aerosol concentrations in animal confinement buildings are dependent on animal activity, air temperature, relative humidity, ventilation rate, animal stocking density, animal mass, type of litter, type of bird, bird age, type of feed, feeding method, time of day, air distribution, relative locations of dust sources and presence or absence of air cleaning technologies [6, 7].

Microorganisms exist suspended in the air as well as attached to dust particles. The survival time for bacteria is affected by many factors: mechanism of dispersal into the air, deposition on host surfaces, host susceptibility, humidity, temperature, bacterial repair processes and the open-air factor, which can kill microorganisms. Therefore, management practices can directly affect the levels of bacteria. For example, increasing the stocking density and temperature of poultry facilities leads to an increase in the concentrations of airborne organisms [7].

Vents located along the walls and in the roof allow for outdoor air intake. Circulating fans move the air throughout the barn while ventilation fans move air across the barn. Contaminated indoor air is expelled from animal facilities by exhaust fans. *E. coli* and *Salmonella* were isolated up to 12m from poultry facilities. At 3m from poultry building exhaust fans, dust concentrations can be relatively high (32-75mg/m^3) but fall below 2mg/m^3 by 12m from ventilation fans [5]. An increased ventilation rate will not necessarily reduce overall dust concentrations within the building since the dust production rate increases with increased ventilation. Dust levels depend on relative humidity. Less ventilated buildings have high relative humidity and lower dust aerosolization than highly ventilated buildings. However, in buildings with natural ventilation or extremely high ventilation rates, dust levels drop [8]. Increasing relative humidity to 75% has been shown to lower inhalable dust (the fraction that is below 20μm), but not respirable dust (the fraction below 5μm) [6]. However, litter moisture increases during periods of high humidity and ammonia levels increase with litter moisture [6].

Mechanical disturbance by bird movement is the prime method of aerosolization in poultry facilities. If light programs are used, dust concentrations are much lower at night than during the day due to less animal movement [6]. Aerosolization of organic dust particles and endotoxin varies between the two poultry barn types. There is less ground disturbance in facilities where birds are not housed on the floor and movement is restricted.

The type of flooring and litter used in the facility alters aerosolization of dust particles [5]. Generally, dust concentrations are lowest in cage-housed facilities that use manure collection systems and are highest in floor-housed operations that use litter as bedding material. At 32ºC, the rate of dust production in floor-housed operations decreases to the rate observed in cage-housed facilities. This is attributed to an increase in humidity, which decreases the generation rate of dust from floor litter and causes airborne dust to settle more rapidly [7]. There is a predominance of female birds as well as different bird types in cage-housed versus floor-housed operations. In floor-housed operations it is expected that aerosolization of dust increases throughout the chicken growth cycle [4]. Young birds undergo molting, which contributes to large particle production during this time of development. Birds enter floor-housed operations at approximately 7 days of age and are removed by approximately 40 days of age. However, birds enter cage-housed facilities at approximately 20 weeks of age and continue laying eggs until approximately 70 weeks of age. These differences coincide with observations of greater dust concentrations in floor-housed poultry facilities.

Many management practices have been identified that influence aerosolization and dispersal of dust (Figure 1) [3]. Using optimal practices for lowering aerosolization is a potential means for lowering dust exposure in poultry operations.

1.1.2.3 Exposures

Aerosolization of dust particles into the breathing zone of workers results in exposure to bioaerosols. Poultry farmers are exposed to higher levels of environmental contaminants, such as dust, endotoxin, and microorganisms, than other agricultural workers [50]. Dust concentrations in poultry facilities can range from 0.02 to 81.33mg/m^3 for inhalable dust and 0.01 to 6.5mg/m^3 for respirable dust. Cage-housed facilities show the lowest dust concentrations, <2mg/m^3, while dust concentrations in floor-housed operations are typically four to five times higher [6]. Reported endotoxin concentrations in poultry houses range from 0.2 to 9.5 x 10^3 EU/m^3 [1, 4, 51, 52]. Endotoxin levels are also typically higher in cage-housed versus floor-housed operations [1]. Endotoxin concentrations in respirable dust, 20 to 40ng/mg, are considerably higher than endotoxin concentrations in total dust, 6 to 16ng/mg, suggesting that endotoxin is enriched in smaller particles [53]. The following exposure limits have been proposed: 2.4 or 2.5mg/m^3 for total dust, 0.16mg/m^3 or 0.25mg/m^3 for respirable dust, 61ng/m^3 or 600 EU/m^3 for endotoxin, and 12ppm ammonia [8, 54].

Typically, airborne microorganisms are reported as CFU/m^3 air. Culturable airborne bacteria concentrations in poultry environments range from 9 x 10^4 to 7 x 10^5 CFU/m^3 [55] and culturable airborne fungal spores range from 7 x 10^3 to 4.9 x10^4 CFU/m^3 [21]. Culturable bacteria are most concentrated in 0.65 to 1.1μm bioaerosols and

culturable fungi are most concentrated in 2.1 to 3.3μm bioaerosols [56]. Recent results show that culture-dependent techniques underestimate total bacteria or total fungi measured by culture-independent approaches such as quantitative PCR [57]. The measure of total fungi in poultry operations is $2.0x10^7/m^3$ and measures of total bacteria range from 4.6×10^5 to 4.2×10^{10} bacteria/m^3 [4, 50, 58]. There are no threshold limit values for total bacteria but the suggested occupational exposure of culturable bacteria is 10^5 CFU/m^3 air [55]. Airborne archaea have been detected in bioaerosols from dairy and swine barns at concentrations of 10^4 to 10^6 archaea/m^3 and 10^6 to 10^8 archaea/m^3, respectively [28, 29]. Airborne archaea have never been detected in bioaerosols from poultry houses and no safe occupational exposure levels have been set.

Dust is a complex mixture of both viable and non-viable sources, including endotoxin, bacteria, fungi, and archaea. Therefore, monitoring of several types of exposures is necessary. Characterizing typical exposure levels to each of these contaminants is required to help set exposure limits and to find potential means of lowering exposures for remediation.

1.1.2.4 Responses

The following lung function measurements are used during the assessment of respiratory health: forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), and forced expiratory flow rate between 25 and 75% of FVC (FEF_{25-75}). Decreases in FEV1, FVC and FEF_{25-75} are normally indicative of obstructive ventilation caused by narrowing of the airways. Cross-shift declines in FEV_1, FVC and FEF_{25-75} have been identified and correlate to endotoxin exposure in the workplace [59]. Cross-

shift changes have also been shown to predict longitudinal changes in lung function [60]. Restrictive disorders are caused by changes in compliance of lung tissues or the chest wall [7]. Several techniques are used to study the lower respiratory tract. Methacholine challenge induces airway hyperresponsiveness. Bronchoscopy, including bronchoalveolar lavage (BAL), is used to compare symptomatic and asymptomatic workers [59].

1.1.2.4.1 Worker symptoms

Exposure to endotoxin causes episodic febrile reactions. Toxin fever generally occurs in the afternoon or evening of a working day. Symptoms of toxin fever include: headache, nausea, coughing, nasal irritation, chest tightness and phlegm. The minimum level of endotoxin required to produce a fever reaction in humans is 0.5μg/m^3 following a four-hour exposure period [7]. Endotoxins derived from different species of Gram-negative bacteria differ in their toxicity. Therefore, the minimum level required to produce fever is species-dependent. Inhalation of endotoxin can cause many physiological airway responses including airflow obstruction, airway hyperreactivity and a reduction in alveolar diffusion capacity. BAL fluid following endotoxin instillation shows increased numbers of macrophages and neutrophils along with increased concentrations of interleukin-6 (IL-6), IL-8, IL-1β, and tumor necrosis factor (TNF-α) [61].

Exposure to the confinement barn environment can cause acute and chronic respiratory symptoms in workers similar to those observed following endotoxin inhalation. Studies of nine different industries showed the highest prevalence of work-

related lower and upper respiratory symptoms and chronic bronchitis in poultry workers [1]. Clinical diseases observed in poultry workers include allergic and non-allergic rhinitis, organic dust toxic syndrome (ODTS), chronic bronchitis, hypersensitivity pneumonitis (Farmer's Lung), toxin fever and occupational asthma or asthma-like syndrome [7, 50, 54, 59, 62]. Malaise, myalgias, chest tightness, headache and nausea are characteristic symptoms of ODTS following exposure to organic dust. Symptoms typically arise within 4 to 8h following exposure and can last for several days. These symptoms are self-limiting but can result in heightened risk of chronic bronchitis [59].

Poultry workers typically complain of chronic cough that may be accompanied by phlegm, eye irritation, fatigue, headache, nasal congestion, fever, throat irritation, chest tightness, shortness of breath and wheezing [1, 52, 63]. These asthma-like symptoms are associated with number of hours worked per day [59]. Significant differences in symptoms are observed between cage-housed and floor-housed workers. Current and chronic phlegm occurred more frequently in workers from cage barns. Endotoxin concentration (EU/mg) is shown to be a significant predictor of chronic phlegm [1]. However, the symptoms generated by poultry dust are thought to be non-specific and caused by a variety of agents, which makes it difficult to find a dose-response relationship or set exposure limits [7].

Naïve subjects exposed to the barn environment have been shown to develop symptoms such as: cough, dyspnea, nasal stuffiness, headache, fever and chills, malaise, nausea and eye irritation after several hours of exposure. Following acute exposure, naïve subjects also show airway hyperresponsiveness characterized by a decline in peak expiratory flow rates and FEV_1 [59]. Continued exposure for only a short period of time

(weeks) can increase bronchial hyperresponsiveness and lead to occupational asthma. The "healthy worker effect" is the phenomenon where individuals affected by symptoms leave the industry following only a short exposure period [64]. Therefore, only less sensitive workers remain in the industry long-term.

1.1.2.4.2 Airway inflammation

Animal confinement workers typically suffer from airway inflammation that is characterized by the presence of increased numbers of neutrophils and macrophages but not eosinophils, as is observed in asthma [59]. Recruitment of resident macrophages and neutrophils has been demonstrated in BAL fluid following inhalation of LPS [13]. A likely mechanism for the accumulation of inflammatory cells following airborne contaminant exposure is an increased production of proinflammatory cytokines and chemokines from structural cells such as epithelial cells [59]. Analysis of nasal lavage fluid following 24h endotoxin exposure reveals the presence of several inflammatory cytokines and chemokines including: interleukin-6 (IL-6), IL-8, IL-1α and IL-1β [59]. Increased concentrations of inflammatory mediators IL-8, IL-1β, and tumour necrosis factor (TNF-α) have been recovered from BAL fluid following LPS challenge [13]. Macrophages and bronchial epithelial cells produce IL-8 in response to challenge with organic dust [59]. IL-8 is a specific neutrophil chemoattractant and IL-1β and TNF-α induce endothelial adhesion molecule production, which is necessary for neutrophil recruitment [59]. Following leukocyte migration to the local target site, these cells release their own chemoattractant mediators, expanding the inflammatory response [11]. Airway epithelial cells express intercellular adhesion molecule (ICAM-1), which is

modulated by bacterial products, such as LPS, and inflammatory mediators, such as interferon (IFN-γ) [65]. Exposure to barn air results in increased soluble ICAM-1 in BAL fluid and enhanced adhesion of lymphocytes to the epithelium [65]. The distribution of inflammatory cells is different between workers and naïve subjects, suggesting that the mechanisms leading to chronic disease are different than those leading to the acute response in control subjects [59].

Thickening of the reticular basement membrane during airway repair occurs following endotoxin exposure. IFN-γ and LPS induce airway epithelial cells and macrophages to produce defensins, and collectins (Surfactant Protein A) [17]. In rats, intratracheal LPS exposure after 16 days results in increased extracellular matrix and altered secretion of surfactant by newly differentiated type II pneumocytes [13]. An animal model of long-term endotoxin exposure shows airway changes similar to those observed in humans with chronic bronchitis, including airway wall thickening [59].

Mucus coats the luminal surface of the airway and acts as a protective barrier against toxins and pathogens. This mucus barrier is an important component of the innate immune system of the lungs, clearing particles and infectious agents from the airways. Water, ions, proteins, lipids, and glycoproteins are the main components of mucus. Mucus from healthy individuals is composed of 40–50% mucin glycoprotiens, by weight [66]. With chronic infection and/or inflammation, cells and cellular components are included in mucus, creating sputum that contains leukocytes, DNA, proteoglycans, and filamentous actin. Mucins are present at high levels in sputum of patients with cystic fibrosis, chronic bronchitis, and asthma [66]. *MUC2* and *MUC5AC* gene expression is upregulated by LPS and other bacterial components [66]. Several inflammatory

cytokines and chemokines regulate mucin gene expression. TNF-α, IL-1β, and IL-9 upregulate *MUC2* and *MUC5AC* mRNA expression [66]. IL-6 and IL-17 increase expression of *MUC5AC* and *MUC5B* [66].

Airway inflammation likely contributes to the development of respiratory symptoms such as cough and a decline in lung function observed in workers. Dust, endotoxin and ammonia have all been associated with chronic respiratory illness in swine workers. Endotoxin is an important component of hog barn dust and is recognized as a primary inflammatory stimulus in grain dusts. However, hog dust extract (HDE) was a more potent stimulus of airway epithelial cell IL-8 and IL-6 release than LPS alone, at the same concentration [67]. LPS, in the same concentration as the amount of endotoxin measured in HDE, does not augment lymphocyte adhesion [65]. These observations suggest that endotoxin alone is not solely responsible for hog barn dust-induced airway inflammation. LPS and other immunostimulatory bacterial components, such as peptidoglycan and bacterial DNA, signal through different pathways, providing a reason for more potent immune stimuli by barn dust extract than by LPS alone [17]. It is possible that changes in epithelial cell cytokine release are regulated by many bioaerosol components.

1.1.2.4.3 Toll-like receptor signaling

Toll-like receptors (TLRs) specifically recognize different microbial molecules and are important for innate immune system activation [14]. The innate immune system is the first line of host defense involved in recognizing invading microbial pathogens. Ten distinct TLRs have been identified in humans [68]. Some TLRs reside at the cell

surface (1, 2, 4 and 6) while others are found within the cell, mainly within the endoplasmic reticulum (3, 7, 8 and 9) [69]. Intracellular TLRs typically detect nucleic acids of invading pathogens.

Among all TLRs, TLR4, which recognizes LPS, has been the most intensively studied. Most LPS moieties activate cells through binding TLR4. However, LPS from some bacterial species, such as *P. gingivalis*, activate cells through TLR2 binding [70]. LPS-binding protein is the first molecule involved in LPS recognition. LBP binds to LPS and forms a complex with LPS receptor molecule, CD14. LPS is transferred from this complex onto the LPS receptor complex composed of TLR4 and MD2. Following LPS binding to TLR4, TLR signaling is enhanced by homodimerization of receptor and recruitment of Toll/IL-1R (TIR)-domain-containing proteins to the receptor's cytoplasmic domain. These adaptors include: myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal), TIR-containing adaptor inducing IFNβ (TRIF), and TRIF-related adaptor molecule (TRAM). MyD88-dependent signaling occurs through the activation of the NF-κB, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways. These pathways regulate the balance between cell viability and inflammation [14].

1.1.2.4.4 Immune adaptation

Adaptation occurs when repeated exposures result in a reduced injury response compared to a single exposure alone. There is evidence to support an adaptive response to endotoxin exposure in animal confinement workers. A lower number of inflammatory cells is recovered from the lower respiratory tract of workers compared to naïve subjects

and a smaller decline in lung function and reduced bronchial responsiveness to methacholine is observed in workers versus naïve controls [59]. Tolerance to neutrophil recruitment to the airways and release of IL-1β and TNF-α can be induced by daily challenge with sublethal endotoxin concentrations [59]. Pretreatment with TNF-α can induce endotoxin tolerance and anti-inflammatory cytokine IL-10 plays a role in endotoxin tolerance [59].

Genetic factors, such as TLR mutations, also play a role in endotoxin tolerance. A polymorphism of TLR4 (Asp299Gly) is observed in approximately 10% of individuals in the general population and has been associated with a blunted response to LPS in vitro and with a diminished airway response to inhaled LPS [13]. This missense mutation alters the extracellular domain of the TLR4 receptor. An additional polymorphism (Thr399Ile) co-segregates with the Asp299Gly substitution [70]. Co-segregating missense mutations are associated with hyporesponsiveness to inhaled LPS in humans. However, not all hyporesponsive individuals have TLR4 mutations and not all individuals with TLR4 mutations are hyporesponsive to LPS [71]. These results indicate the importance of other genetic and/or environmental factors in determining response to inhaled endotoxin and a need for further studies to understand the mechanisms.

1.1.2.5 Remediation

The overwhelming evidence of the respiratory symptoms and immunological effects of poultry dust exposure suggests a need for remediation. However, many sources of dust, including some sources of endotoxin, are intrinsic to the poultry production industry and therefore, remediation is difficult (Figure 1) [3]. Keeping poultry facilities

clean has long been encouraged as a method to protect human respiratory health. Adopting management practices such as use of pelleted food, routine entry into buildings and use of lighting cycles can control dust and ammonia levels. However, some practices may lower one contaminant while increasing another. For example, dry litter reduces ammonia production but is aerosolized more easily by animal activity. Also, application of water mists can reduce dust production by increasing the settling velocity of airborne particles but increases relative humidity, which facilitates ammonia production [7].

Both the use of well-fitted N-95 respirators by workers and spraying water or oil mixtures to reduce dust are shown to be effective at reducing dust exposure in animal confinement buildings [6, 8, 11, 72, 73]. The oil binds to dust particles, preventing dispersal and resuspension from surfaces. Although spraying water is useful at reducing dust production, it increases relative humidity, which facilitates microbial growth [7]. Use of well-fitted N-95 respirators over a four-hour exposure has proved to reduce the inflammatory response as measured by elimination of acute respiratory symptoms, shift changes, lung function response to methacholine inhalation, serum IL-6 response and nasal lavage IL-6 and IL-8 responses [73]. Spraying canola oil to reduce dust exposure has shown to be beneficial in reducing declines in lung function following a five-hour exposure in a swine facility [11]. However, long-term health effects of canola oil sprinkling to workers and animals are unknown.

Chronic inhalation of LPS results in an asthma-like response, including airway inflammation, airway hyperreactivity, and airway remodeling. TLR4 antagonists E5531 and E5564, lipid A analogs, prevent the chronic airway response to inhaled LPS [74]. E5564 decreases airway hyperreactivity to methacholine, neutrophilia, IL-6 in the lavage

fluid, and neutrophil infiltration of the airways. E5531 inhibits TLR4/MD2 binding of LPS and it is likely that E5564 acts in the same way. LPS responsiveness is limited in epithelial cells that have low MD2 expression. These observations suggest that competitive LPS antagonists may play a therapeutic role in preventing or reducing endotoxin-induced airway disease.

Altering management practices may be a means of reducing aerosolization of barn contaminants, thus reducing worker exposure. Understanding the levels of worker bioaerosol exposures may help introduce new management practices to reduce exposure, such as better personal protective equipment. Examining the differences in bioaerosols and worker responses between cage-housed and floor-housed poultry operations may provide insight into other means of remediation.

1.1.3 Summary

Dust sources, including endotoxin and bacteria, are present at high concentrations in poultry facilities. The production of poultry dust can vary due to factors including: animal activity, air temperature, relative humidity, ventilation rate, animal stocking density, type of litter, type of bird, bird age, type of feed, feeding method, time of day, air distribution, relative locations of dust sources and presence or absence of air cleaning technologies [6, 7]. Also, particle size is a key factor in poultry dust production since rate of aerosolization, settling velocity and resuspension rate of airborne particles differ depending on particle size [8].

Dust production is typically higher in floor-housed versus cage-housed poultry facilities [1]. Management practices differ between the two types of poultry facilities.

Animal activity is higher in floor-housed operations where birds move freely as opposed to being housed in cages. This higher level of activity contributes to greater particle aerosolization. Litter is a source of dust production and is used in floor-housed operations but not in cage-housed facilities. The predominance of female birds in cage-housed operations as well as different bird types may contribute to differences in the air environment. Bird age is also a factor that differs between the two barn types and has an effect on bioaerosols.

A better understanding of the barn air environment, including bioaerosols, is required to reduce aerosolization and dispersal, decrease worker exposure and prevent or treat respiratory symptoms. Further examination of the aerobiological pathway will help to find means of remediation. The respiratory dysfunction of poultry workers is a major health issue and requires detailed investigation.

1.2 Overview and rationale

In 2011, Canadians consumed more chicken than other common meats [75] and purchased nearly 270 million dozens of eggs [76]. The Canadian poultry industry contributes up to $6.8 billion to the Canadian economy and creates approximately 56,000 jobs [75]. The industry is nation-wide, with facilities in every province. The Saskatchewan poultry industry contributes over $177 million to the Canadian economy and provides roughly 2,500 jobs [75]. In 2011, there were 72 chicken farmers [75] and 75 egg producers [76] registered in Saskatchewan.

Modern methods of poultry facility management require that workers spend a large proportion of the day in an environment containing high levels of dust, gases and

odours [2, 7]. Studies of different industries showed the highest prevalence of work-related symptoms including chronic cough, phlegm, eye irritation, dyspnea, fatigue, headache, nasal congestion, fever, throat irritation, chest tightness and wheezing, and lower baseline lung function in poultry workers [1, 50, 52, 63]. Clinical diseases observed in poultry workers include allergic and non-allergic rhinitis, ODTS, chronic bronchitis, hypersensitivity pneumonitis (Farmer's Lung), toxin fever and occupational asthma or asthma-like syndrome [7, 50, 54, 59, 62]. Although poultry workers from meat production facilities (or floor-housed operations) and egg production facilities (or cage-housed operations) both report respiratory symptoms, cage-housed workers report a higher prevalence of current and chronic phlegm [1]. It is important to understand the sources of bioaerosols from both environments to better develop remediation strategies that lower workplace exposures.

The work presented in this thesis includes analyses of samples previously collected by Kirychuk *et al.* and area dust and endotoxin concentrations have been published [77]. This project compares area and personal bioaerosols from cage-housed and floor-housed poultry operations. Specifically, levels of dust, endotoxin, bacteria, archaea, and antimicrobial resistance genes are examined.

1.3 Research questions and hypotheses

The objective of this research was to characterize biological components of poultry bioaerosols (quantitatively and qualitatively), and to compare the bioaerosols from cage-housed and floor-housed poultry operations. The following questions founded this research program:

1. Do levels of total dust, endotoxin and bacteria differ between cage-housed and floor-housed poultry bioaerosols?

Previous research has shown that levels of total dust are significantly higher in floor-housed facilities but that there is a trend towards higher levels of endotoxin in cage-housed operations.

Hypothesis 1. Dust will be higher in floor-housed bioaerosols. Endotoxin and bacteria will be greater in cage-housed bioaerosols.

2. Does the type of housing facility influence bacterial diversity in poultry bioaerosols?

Cage-housed and floor-housed operations differ in many ways including bird age, bird gender, number of birds, use of litter, antibiotic use, presence of eggs, and CO_2 levels.

Hypothesis 2. Bacterial diversity will differ between cage-housed and floor-housed bioaerosols.

3. Are antimicrobial resistance genes present in poultry bioaerosols? If so, do they differ between cage-housed and floor-housed bioaerosols?

Antibiotics are used in poultry production for several reasons including prophylaxis, treatment, and growth promotion. Antibiotic use differs between cage-housed and floor-housed facilities.

Hypothesis 3. Antimicrobial resistance genes will be present in poultry bioaerosols and at greater levels in floor-housed bioaerosols where growth-promoting antibiotics are used.

4. Are archaea present in poultry bioaerosols? If so, do archaea concentrations differ between cage-housed and floor-housed bioaerosols?

Archaea have been detected in bioaerosols from swine and dairy barns.

Hypothesis 4. Archaea will be detected in poultry bioaerosols and at higher concentrations in cage-housed bioaerosols.

2.0 CHAPTER TWO: Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations

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Author Contributions

All authors participated in the design of the experiments and contributed to writing of the manuscript. The author of this thesis contributed to sample collection, dust measurement, DNA extraction, qPCR, PCR-DGGE and statistical analysis.

2.1 Abstract

Background. Although bioaerosols from both cage-housed (CH) and floor-housed (FH) poultry operations are highly concentrated, workers from CH operations have reported a greater prevalence of respiratory symptoms. *Objective.* The objective of this study was to directly compare bacteria, both quantitatively and qualitatively, in bioaerosols from CH and FH poultry facilities. *Methods.* Bioaerosols were collected from fifteen CH and fifteen FH poultry operations, using stationary area samplers as well as personal sampling devices. Dust, endotoxin and bacteria were quantified and bacterial diversity was investigated using PCR followed by denaturing gradient gel electrophoresis (DGGE). *Results.* Dust ($p<0.001$), endotoxin ($p<0.05$) and bacteria ($p<0.05$) concentrations were significantly higher in personal bioaerosols from FH poultry operations than CH bioaerosols. Although dust and endotoxin concentrations did not differ significantly between area and personal samples within each barn type, clustering analysis of DGGE profiles of bacteria revealed that area and personal samples shared less than 10% similarity. These data suggest that area samples are not representative of personal bacteria exposures, which may be affected by worker movement, bacteria carried on the worker and worker location. Personal DGGE profiles from CH and FH operations shared less than 20% similarity and composite analysis showed that bacteria were more prevalent in personal samples from CH bioaerosols than FH bioaerosols. *Conclusions.* Bacteria concentration and diversity are significantly different between bioaerosols from CH and FH poultry operations.

2.2 Introduction

Poultry facilities are associated with high production of dust, gases and odours [7]. Poultry workers report a higher prevalence of work-related eye, respiratory and skin symptoms than controls [51] and they report a higher prevalence of work-related lower and upper respiratory symptoms than other agricultural workers [78]. Workers typically report chronic cough that may be accompanied by phlegm, eye irritation, dyspnea, fatigue, headache, nasal congestion, fever, throat irritation, chest tightness and wheezing [52]. Clinical diseases observed in poultry workers include allergic and non-allergic rhinitis, organic dust toxic syndrome (ODTS), chronic bronchitis, hypersensitivity pneumonitis (Farmer's Lung) and occupational asthma or asthma-like syndrome [7, 50, 54, 62]. Poultry workers report a greater prevalence of chronic bronchitis and ODTS than other agricultural workers [78]. Although endotoxin, a Gram-negative bacterial cell wall component [13], has been shown to be predictive of respiratory symptoms [78], dust is heterogeneous and other components may contribute to these symptoms.

The type of housing may influence levels of environmental contaminants in poultry dust. There are two common types of poultry housing facilities: cage-housed (CH) operations, where birds are housed in cages for egg production, and floor-housed (FH) operations, where birds are housed on the floor for meat production. There are a number of differences in the two types of poultry operations including: worker time spent in direct contact with birds, predominance of female poultry in CH facilities, presence of eggs in CH operations, presence and type of litter in FH facilities, age of birds, length of time birds spend in housing and housing management practices (including antibiotic use) [3]. Currently, there are few studies that compare CH and FH poultry operations. There

have been reports of greater current and chronic phlegm in workers from CH facilities than workers from FH operations, despite dust levels being significantly higher in FH operations [1, 63, 77].

In poultry bioaerosols, bacteria exist suspended freely in the air as well as attached to dust particles [7]. Studies often focus on detection of culturable airborne bacteria [51, 79]. However, bioaerosol data collected by culture-independent techniques, such as quantitative PCR, suggest that viable counts underestimate levels of biological contaminants [57]. Since the introduction of PCR followed by denaturing gradient gel electrophoresis (DGGE) [80], there have been many studies on bacterial diversity of intestinal, fecal and litter samples from poultry operations [81-84], but not on bioaerosols. Chicken microbiota diversity depends on bird age and antibiotic use [83], characteristics that differ between CH and FH poultry facilities. Also, one study on poultry bioaerosols indicates that airborne bacteria, endotoxin and inhalable dust increase during the chicken growth cycle [4]. Therefore, it is reasonable to expect differences in bacteria concentration and the bacterial diversity of bioaerosols from CH and FH poultry operations. This study will focus on comparing dust, endotoxin and bacteria concentrations as well as bacterial diversity between bioaerosols from CH and FH poultry operations.

2.3 Materials and methods

2.3.1 Bioaerosol sampling

Air sampling was performed by Kirychuk *et al.* at 15 cage-housed (CH) and 15 floor-housed (FH) poultry operations in Saskatchewan, described previously [77].

Briefly, two area (A) samples and one personal (P) sample were collected at each barn. Dust was collected on pre-weighed radial slit polyvinyl chloride (PVC) filters using a Marple cascade impactor (5μm; Thermo Electron Corp., Waltham, MA, USA) connected to a constant airflow pump (Universal 224-PCXR4; SKC, Eighty Four, PA, USA) run at 2L/min over a 4h sampling time. Six stages (3 through 8, with cut-points 0.52, 0.93, 1.55, 3.5, 6.0 and 9.8μm) were included in the Marple sampler. Only results from dust fractions >3.5μm (stages 3, 4 and 5) are reported here.

2.3.2 Dust and endotoxin analysis

Dust and endotoxin were measured by Kirychuk *et al.*, described previously [77]. Briefly, gravimetric analyses were performed to measure dust (MX5 microbalance; Mettler-Toledo, Greifensee, Switzerland). Dust from individual filters was extracted in 10mL sterile, pyrogen-free, endotoxin-free water (LAL reagent water; BioWittaker, Walkersville, MD, USA) and rocked at room temperature for 60min (Labquake shaker; Labindustries, Berkeley, CA, USA). Aliquots of 0.5mL were applied to kinetic-QCL Limulus Amoebocyte Lysate (LAL) assays to quantify endotoxin (*Escherichia coli* O55: B5; Cambrex BioScience Walkersville Inc, Walkersville, MD). Dust concentrations were expressed as milligrams per cubic meter of air (mg m^{-3}) and endotoxin concentrations were expressed as endotoxin units per cubic meter of air (EU m^{-3}).

2.3.3 DNA extraction

Aliquots of 1.5mL extracted dust were centrifuged (10min, 21000g, room temperature) and pellets were stored at -20°C until DNA extraction. Isolation of total

genomic bacterial DNA was performed using the QIAamp DNA Mini Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions for tissue with modifications for bacteria. Total DNA samples were eluted in 100μL elution buffer, supplied with the kit.

2.3.4 Quantitative real-time PCR for bacteria

Amplification was performed using a DNA Engine Opticon2 (Bio-Rad, Mississauga, ON, Canada) and all primers and DNA probes (see Table I) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Bacteria quantification was performed with 16S rRNA forward primer (5'-GGTAGTCYAYGCMSTAAACG-3'), 16S rRNA reverse primer (5'-GACARCCATGCASCACCTG-3') and 16S rRNA probe (FAM-TKCGCGTTGCDTCGAATTAAWCCAC-IB^{TM}FQ), previously described [85]. The PCR components were as follows: 2.5μL DNA template, 0.4μM each primer, 0.08μM probe, 0.5U uracil N-glycosylase (UDG) (Sigma, Oakville, ON, Canada) and 12.5μL 2X QuantiTect Probe PCR kit (Qiagen, Mississauga, ON, Canada). The PCR program was as follows: hold at 37°C for 10min, hold at 95°C for 15min then 40 cycles of 95°C for 20s and 62°C for 60s. Quantification of a standard curve prepared from *E. coli* genomic DNA was performed. Data were collected with the Opticon Monitor software 2.02.24 and analyzed by linear regression $\log_{10}$(copy number)=f(threshold cycle). PCR efficiency was determined by $E=10^{(-slope)}-1$. The absolute 16S rRNA copy number was multiplied by 1.75 (the ratio of 16S rRNA copies in *E. coli* to the average of all bacteria) to determine the number of bacteria in each sample. Field blanks and negative controls were included to detect PCR reagent contamination.

Table I. Primers and probes

Primer	Target	Sequence	Reference
EUBf	Bacteria 16S rRNA	GGTAGTCYAYGCMSTAAACGT	Bach *et al*. 2002
EUBr	Bacteria 16S rRNA	GACARCCATGCASCACCTG	Bach *et al*. 2002
EUBp	Bacteria 16S rRNA	FAM-TKCGCGTTGCDTCGAATTAAWCCAC-IB^{TM}FQ	Bach *et al*. 2002
341f*	Bacteria 16S rRNA	CCTACGGGAGGCAGCAG	Muyzer *et al*. 1993
518r	Bacteria 16S rRNA	ATTACCGCGGCTGCTGG	Muyzer *et al*. 1993
907r	Bacteria 16S rRNA	CCGTCAATTCCTTTGAGTTT	Yu and Morrison 2004

FAM, 6-carboxyfluorescein; IB^{TM}FQ, Black Hole Quencher-1 (Integrated DNA Technologies, Coralville, IA, USA)
All primers were purchased from IDT (Coralville, IA, USA)
* GC-clamp, CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer *et al*. 1993)

2.3.5 Denaturing gradient gel electrophoresis (DGGE) analysis

In order to produce bacterial DGGE profiles for each sample, DNA from stages 3, 4 and 5 were pooled, as well as both area samples, prior to DGGE analysis. Therefore, 60 samples (15 CH area, 15 CH personal, 15 FH area and 15 FH personal) were characterized. For bacterial diversity and clustering analysis, the variable V3 region of 16S rRNA gene from nucleotide 341 to 534 (*E. coli* sequence) was amplified using GC-341f (5'-CCTACGGGAGGCAGCAG-3') and 534r (5'-ATTACCGCGGCTGCTGG-3') primers (Table I). The PCR program was used as described previously [80]. The PCR components were as follows: 0.5μM each primer, 5μL DNA and 1.25U Taq DNA polymerase (Promega, Madison, WI, USA). For sequencing analysis, the variable V3 region of 16S rRNA gene from nucleotide 341 to 907 (*E. coli* sequence) was amplified using GC-341f (5'- CCTACGGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') primers (Table I). The PCR program was performed as described previously [86]. The PCR components were as follows: 0.5μM each primer, 5μL DNA and 1.25U Taq DNA polymerase (Promega, Madison, WI, USA). PCR was performed by the DNA Engine DYAD^{TM} thermocycler (Bio-Rad, Mississauga, ON, Canada). Following 1.5% agarose gel electrophoresis, DNA was quantified by comparing band intensities to the EZ Load Precision Molecular Mass Ruler (Bio-Rad,

Mississauga, ON, Canada) measured with GeneTools software (SynGen, Cambridge, England).

Amplified 16S rRNA gene sequence profiles were produced by DGGE as described previously [80, 86] using the DCode (Bio-Rad, Mississauga, ON, Canada). PCR products (60ng) were loaded on 6% (Yu and Morrison primers) or 8% (Muyzer primers) polyacrylamide gels in 0.5X TAE buffer (Bio-Rad, Mississauga, ON, Canada) with a 30-55% denaturing gradient (100% denaturant was 7M urea and 40% v/v deionized formamide). Electrophoresis was performed at 60V for 16h at 60°C. Gels were stained for 15min in 0.5X TAE with SYBR Gold (Molecular Probes, Eugene, OR, USA) and destained for 15min. Gel images were obtained with the imaging system ChemiGenius 2 and GeneSnap software (SynGen, Cambridge, England).

DGGE profiles were normalized and compared by hierarchal clustering using Fingerprinting II INFORMATIX Software 3.0 (Bio-Rad, Mississauga, ON, Canada). All DGGE gel images were matched using a custom molecular DGGE ladder, as described previously [87]. DGGE profile similarity was determined with the Pearson product-moment correlation coefficient and clustering was performed with the unweighted pair-group method using arithmetic averages (UPGMA), using a 1% tolerance.

DNA was excised from bands in the gels using micropipette tips, which were placed in tubes containing aliquots of PCR reagents (Yu and Morrison primers) for 5min. Re-amplification was performed as described above, minus the GC clamp on the forward primer. The PCR products were visualized by agarose gel electrophoresis and sequenced on both strands (CHUL Research Center, Québec, QC, Canada). Each DNA sequence was compared to the Genbank database from the National Center of Biotechnology

Information using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/). Composite analysis of DGGE profiles was performed using Fingerprinting II INFORMATIX Software 3.0 (Bio-Rad, Mississauga, ON, Canada).

2.3.6 Statistical analysis

Dust, endotoxin and bacteria concentrations are reported as raw data. However, log transformation of the data was required prior to statistical analyses. The following comparisons were made using unpaired Student's *t*-tests: FH area vs. CH area, FH personal vs. CH personal, FH area vs. FH personal and CH area vs. CH personal. Results with p-values < 0.05 were considered significant.

2.4 Results

2.4.1 Dust and endotoxin concentrations

Dust concentration was measured using gravimetric analysis. Average dust concentrations for FH and CH area samples were 4.5 and 1.8 mg m^{-3}, respectively [77], while average dust concentrations for FH and CH personal samples were 5.1 and 1.9 mg m^{-3}, respectively. Area and personal dust concentrations were significantly higher in bioaerosols from FH poultry operations than CH bioaerosols ($p<0.001$) (Figure 1A). There was no significant difference between area and personal dust concentrations, within each barn type (FH: $p=0.50$, CH: $p=0.94$).

Endotoxin concentration was evaluated using the *Limulus* Amoebocyte Lysate (LAL) assay. Average endotoxin concentrations for FH and CH bioaerosols, using area samplers, were 2.9 and 1.9 x 10^3 EU m^{-3}, respectively. Average endotoxin concentrations

for FH and CH bioaerosols, using personal samplers, were 3.4 and 2.0 x 10^3 EU m^{-3}, respectively. Endotoxin concentration was significantly higher in FH bioaerosols versus CH bioaerosols collected with personal samplers ($p<0.05$) but did not differ significantly between FH and CH bioaerosols collected with area samplers ($p=0.07$) (Figure 1B). Endotoxin concentrations were not significantly different between area and personal bioaerosols, within each barn type (FH: $p=0.40$, CH: $p=0.56$).

2.4.2 Bacteria concentrations

Bacteria concentration was determined using quantitative real-time PCR. Average bacteria concentrations for FH and CH area bioaerosols were 2.6 and 1.1 x 10^7 bacteria m^{-3}, respectively. Average bacteria concentrations for FH and CH personal bioaerosols were 3.1 and 1.6 x 10^7 bacteria m^{-3}, respectively. Area and personal bacteria concentrations were significantly higher in bioaerosols from FH poultry operations than CH bioaerosols ($p<0.01$ and $p<0.05$, respectively) (Figure 1C). Again, no significant difference in bacteria concentration was observed between area and personal bioaerosols, within each barn type (FH: $p=0.94$, CH: $p=0.65$).

2.4.3 DGGE analysis

Bacterial diversity of poultry bioaerosols was determined using 16S rRNA gene-targeted PCR-DGGE analysis. DGGE profiles, identified with a 1% tolerance, were used for clustering analysis (Figure 2). Interestingly, a similarity of <10% was detected between area and personal DGGE profiles, regardless of barn type. There was <20% similarity between cage and floor personal DGGE profiles. Among area DGGE profiles,

the distinction between cage and floor was less obvious. There was a cluster of 8 floor DGGE profiles (16-23A) that shared <30% similarity with other area DGGE profiles. A single cage DGGE profile (1A) shared <50% with the remaining area DGGE profiles. Among these remaining area DGGE profiles, there were three clusters. One of these clusters consisted of both cage and floor DGGE profiles (2-4A, 14-15A and 24-25A). One cluster contained the remaining cage DGGE profiles (5-13A) and another cluster consisted of the remaining floor DGGE profiles (26-30A).

Bacterial species within poultry bioaerosols were identified using DGGE analysis followed by sequencing. Sequences matching the following bacterial affiliations were detected: *Methylobacterium* sp., *Cupriavidus metallidurans*, *Staphylococcus cohnii*, *Brachybacterium* sp., uncultured Firmicutes, *Mycobacterium* sp., *Brevibacterium avium*, *Megamonas hypermegale*, *Sphingomonas* sp., *Methyloversatilis universalis* and *Nesterenkonia flava* (Table II). All sequences had high similarity (≥97%) with affiliated bacteria. Major bands present in multiple DGGE profiles were sequenced to confirm their affiliation to the same microorganism.

Table II. Closest affiliations to sequences obtained from DGGE profiles of area and personal bioaerosols from cage-housed and floor-housed poultry operations

DGGE band affiliation (accession number)	**bp**	**% similarity**
Methylobacterium sp. (DQ531637.1)	510	100
Cupriavidus metallidurans (FJ644635.1)	514	97
*Staphylococcus cohnii (*GQ169065.1)	520	97
Brachybacterium sp. (GU064364.1)	512	98
uncultured Firmicutes (CU926587.1)	501	98
Mycobacterium sp. (EU360181.1)	515	99
*Brevibacterium avium (*NR_026485.1)	512	99
*Megamonas hypermegale (*FJ489248.1)	532	99
Sphingomonas sp. (AB461702.1)	517	99
*Methyloversatilis universalis (*DQ923115.1)	534	99
*Nesterenkonia flava (*EF680886.1)	514	99

Composite analysis was performed to detect the presence or absence of major bands in all DGGE profiles. When using area samplers, *Mycobacterium* sp., *Brevibacterium avium*, *Cupriavidus metallidurans* and *Brachybacterium* sp. affiliated sequences were more frequently observed in FH bioaerosols than CH bioaerosols (Figure 3A). Uncultured Firmicutes, *Megamonas hypermegale*, *Methyloversatilis universalis* and *Nesterenkonia flava* sequence affiliations were more prevalent in CH bioaerosols than FH bioaerosols. When using personal samplers, *Staphylococcus cohnii* and *Sphingomonas* sp. affiliated sequences were more frequently observed in FH bioaerosols than CH bioaerosols (Figure 3B). The remaining sequence affiliations (*Methylobacterium* sp., *Cupriavidus metallidurans*, *Brachybacterium* sp., uncultured Firmicutes, *Mycobacterium* sp., *Brevibacterium avium, Megamonas hypermegale*, *Methyloversatilis universalis* and *Nesterenkonia flava*) were more prevalent in CH bioaerosols than FH bioaerosols. *Brachybacterium* sp., *Megamonas hypermegale* and *Nesterenkonia flava* affiliated sequences were not detected in floor personal samples.

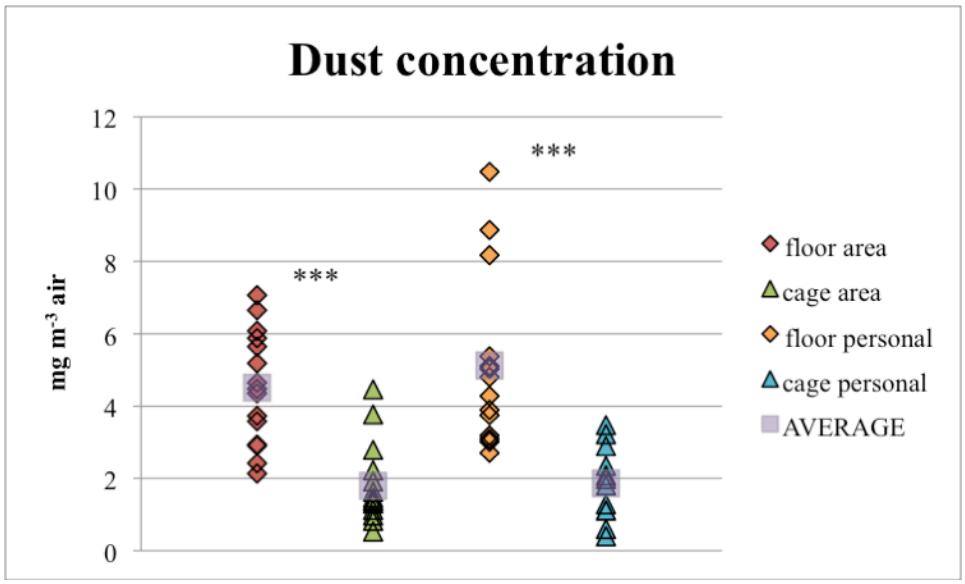

Figure 1A. Dust concentration. Quantification of airborne dust by gravimetric analysis in cage-housed (CH) and floor-housed (FH) bioaerosols collected using area (A) and personal (P) sampling devices. Each point on the graph represents raw data from a single barn, averages indicated. Data was log transformed prior to statistical analyses. ***p<0.001 Area dust concentrations were published previously [77].

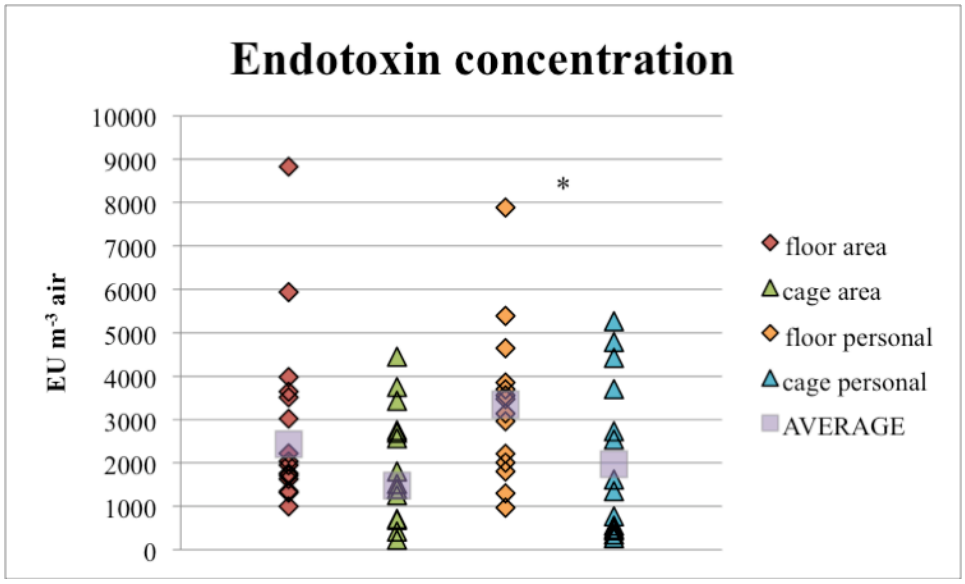

Figure 1B. Endotoxin concentration. Quantification of airborne endotoxin by Limulus Amoebocyte Lysate (LAL) assay in cage-housed (CH) and floor-housed (FH) bioaerosols collected using area (A) and personal (P) sampling devices. Each point on the graph represents raw data from a single barn, averages indicated. Data was log transformed prior to statistical analyses. *p<0.05 Area endotoxin concentrations were published previously [77].

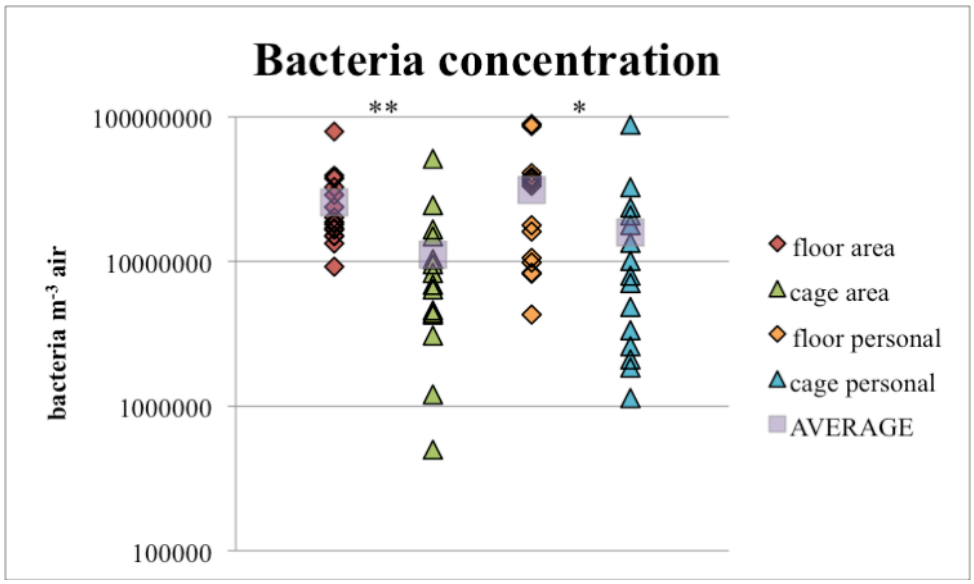

Figure 1C. Bacteria concentration. Quantification of airborne bacteria by real-time PCR in cage-housed (CH) and floor-housed (FH) bioaerosols collected using area (A) and personal (P) sampling devices. The absolute 16S rRNA copy number in each sample, obtained from an *E. coli* standard curve, was multiplied by 1.75 (the ratio of 16S rRNA copies in *E. coli* to the average of all bacteria). Each point on the graph represents raw data from a single barn, averages indicated. Data was log transformed prior to statistical analyses. *$p<0.05$ **$p<0.01$

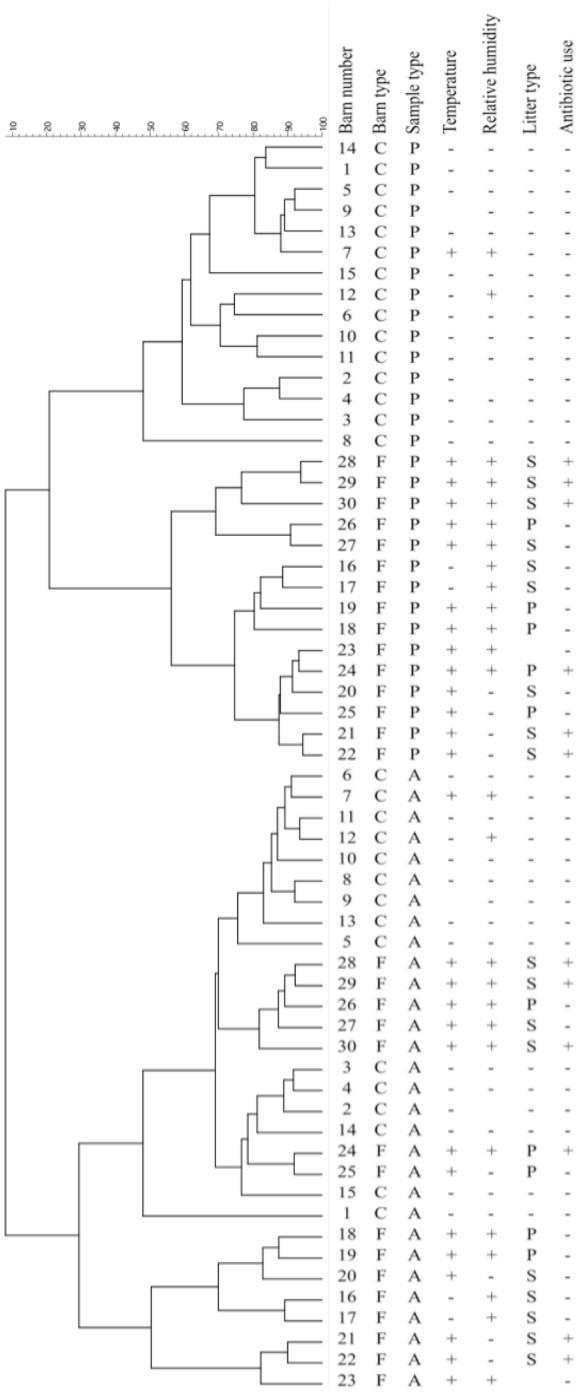

Figure 2. UPGMA (unweighted pair-group method using arithmetic averages) dendogram of bacterial denaturing gradient gel electrophoresis (DGGE) profiles generated via clustering analysis. The scale indicates percent similarity between samples. Samples are of bioaerosols from 15 cage-housed (C, 1-15) and 15 floor-housed (F, 16-30) poultry operations using area (A) and personal (P) sampling devices. Above or below average temperature and relative humidity is indicated (+ or -). Straw (S), paper (P) or no (-) litter is indicated as well as antibiotic use (+ or -). A blank space indicates that information was unavailable.

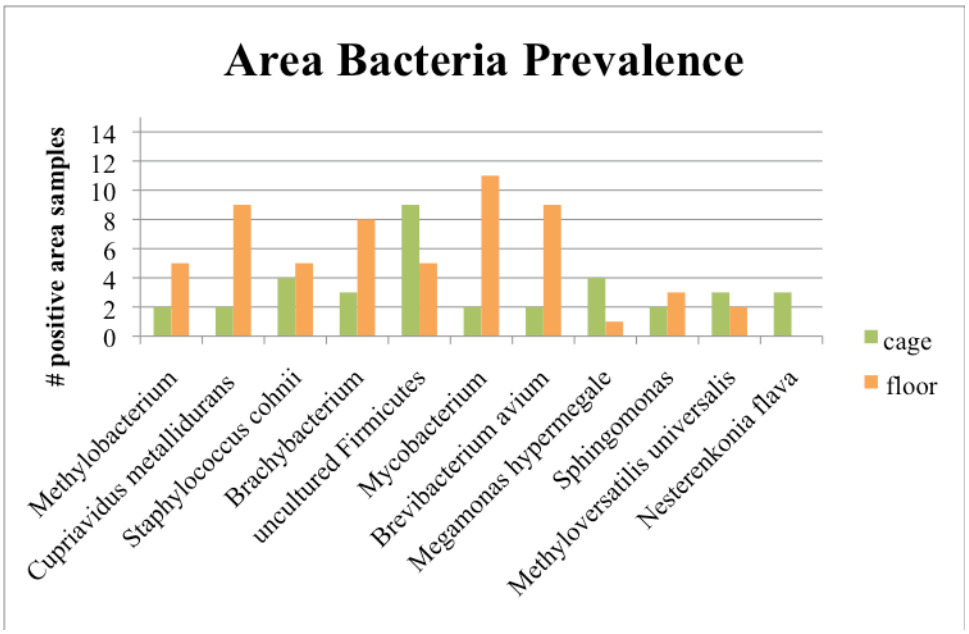

Figure 3A. Area bacteria prevalence. The number of samples positive for each bacterium was determined by composite analysis. Results from cage-housed and floor-housed operations are reported.

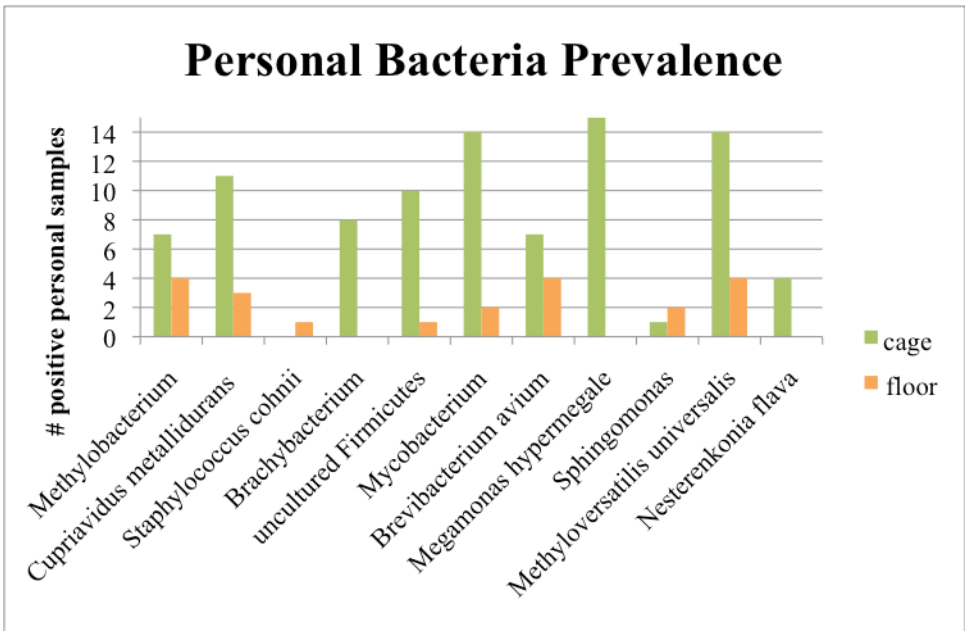

Figure 3B. Personal bacteria prevalence. The number of samples positive for each bacterium was determined by composite analysis. Results from cage-housed and floor-housed operations are reported.

2.5 Discussion

A broad range of poultry dust concentrations has been observed, from as low as 0.02 mg m^{-3} to as high as 81.33 mg m^{-3} [52]. Typically, dust concentrations are 4-5 times higher in FH than CH operations [6]. In this study, dust concentrations ranged from 0.4 to 10.5 mg m^{-3} and were approximately 3-fold higher in bioaerosols from FH facilities than CH operations. Reported endotoxin concentrations in poultry houses range from 0.2 to 9.5 x 10^3 EU m^{-3} [1, 4, 51, 52]. Endotoxin concentrations obtained (0.2 to 8.8 x 10^3 EU m^{-3}) were within published values. Airborne bacteria concentrations in previous poultry studies range from 4.6 x 10^5 to 4.2 x 10^{10} bacteria m^{-3} [4, 50, 58]. Here, airborne bacteria ranged from 5.0 x 10^5 to 8.9 x 10^7 bacteria m^{-3}, within reported values.

It was originally hypothesized that dust, endotoxin and/or bacteria concentrations would be higher in CH bioaerosols than FH bioaerosols to help explain the greater prevalence of respiratory symptoms in workers from CH facilities. However, dust, endotoxin and bacteria concentrations are greater in bioaerosols from FH operations. The high levels of contaminants in FH bioaerosols are supported by previous data that show the increase of these contaminants during the poultry growth cycle [4]. Recently, it has been shown that Gram-negative and Gram-positive bacteria invoke different sets of inflammatory cytokines [19]. The impact of specific bacterial species on lung inflammation is not yet understood. Therefore, it was hypothesized that bacterial diversity, rather than total quantity, may help to explain the greater prevalence of current and chronic phlegm in workers from CH operations. Greater knowledge of the bacterial content of bioaerosols in various occupational settings is essential in order to better understand the health effects of exposed humans.

Clustering analysis showed that area and personal DGGE profiles shared less than 10% similarity, regardless of barn type. It has been previously shown that area and personal samples are not highly correlated [88]. Airflow around a human body differs from airflow around a stationary sampler and worker movement affects bioaerosol dispersal [89]. Personal samples are also influenced by individual factors including lifestyle, health, hygiene, etc. In cage barns, the difference between area and personal DGGE profiles can also be explained by the location of the sampling devices. Workers from CH operations wore personal sampling devices and spent the majority of their time in egg collection rooms adjacent to the barns, while area samplers were set-up in the barns. However, workers from FH facilities wore personal sampling devices while working in the barns. This demonstrates the difficulty of choosing the best area sampling location for exposure assessment purposes.

Types and levels of bacteria are known to depend on management practices that control relative humidity, temperature and litter type [7]. In most cases, we observed that DGGE profiles from barns with similar characteristics, such as above or below average relative humidity, above or below average temperature and paper or straw litter [77], clustered together. Since CH operations typically have lower temperatures, lower relative humidity and do not use litter, these characteristics may, in part, be responsible for the bacterial diversity between CH and FH bioaerosols.

Among personal samples, clustering analysis revealed that CH and FH DGGE profiles shared less than 20% similarity. The observed differences between cage and floor personal samples support the hypothesis that bacterial diversity may be one factor involved in the difference in respiratory symptoms between workers from CH and FH

operations. With the exception of *Sphingomonas* sp. and *Staphylococcus cohnii*, a greater prevalence of all bacteria in cage personal samples was observed (*Mycobacterium* sp., *Methylobacterium* sp., *Brevibacterium avium*, *Brachybacterium* sp., Firmicutes, *Cupriavidus metallidurans*, *Methyloversatilis universalis*, *Nesterenkonia flava* and *Megamonas hypermegale*). Although *Mycobacterium* have not been reported previously in poultry bioaerosols, environmental opportunistic mycobacterial bioaerosols from metalworking fluids, swimming pools, hot tubs and water-damaged buildings are reported to cause respiratory diseases, including hypersensitivity pneumonitis [90-92]. *Methylobacterium* spp. are commonly isolated in the dairy industry [93] but this is the first known detection of *Methylobacterium* sp. in the poultry industry. These bacteria rarely cause human disease but are thought to be opportunistic pathogens. *Brevibacterium* spp. are Gram-positive aerobes that are frequently found in dairy milk, swine bioaerosols and as human skin residents [79, 94]. Recently, *Brevibacterium* spp. have been implicated in human disease [94].

Most microbial identification studies have been performed using culture-dependent techniques, which can overlook those bacteria that are difficult to culture and do not detect non-viable bacteria. In addition, bioaerosol sampling induces significant stress on bacterial cells, reducing their ability to grow in culture. Therefore, the number of bacteria is underestimated using culture-dependent approaches. Some components of non-culturable or non-viable bacteria are known to be immunostimulatory [13] and thus, culture-independent techniques, such as the methods used in this study, are essential for comprehensive bacterial diversity studies. This study is limited by the methodology of sequence identification. DGGE followed by sequencing uses a relatively short DNA

sequence, which could result in less precise sequence identification. Also, the high number of PCR cycles required prior to DGGE is known to decrease sequence diversity [92]. The affiliated sequences detected in this study are, by no means, exhaustive and relevant bacteria may be overlooked. This study provides qualitative data of bacterial diversity and can only draw conclusions on bacteria concentration.

The presence of specific bacteria, including *Jeotgalicoccus* spp. [58] and *Staphylococcus* spp. [4], has been examined in poultry bioaerosols. To the authors' knowledge, this is the first report examining bacterial diversity of bioaerosols from poultry operations. To date, DGGE has been used to look at bacterial diversity in litter samples, microbiota of chickens and bioaerosols from swine confinement buildings. A recent study detected *Brevibacterium avium*, *Brachybacterium* sp. and *Staphylococcus* sp. in poultry litter using PCR-DGGE [84], supporting our observation of these bacteria in poultry bioaerosols. This is also the first known comparison of bacterial quantity and diversity between CH and FH poultry facilities. Very little information exists that compares the two types of poultry operations so this work is an important contribution.

2.6 Conclusions

Biological contaminants are high in poultry bioaerosols, which may help to explain the high prevalence of respiratory symptoms in poultry workers. Dust, endotoxin and bacteria concentrations are higher in FH bioaerosols than CH bioaerosols, which does not help to explain the greater prevalence of respiratory symptoms in workers from CH facilities. Personal DGGE profiles from CH bioaerosols and FH bioaerosols share less than 20% similarity. Personal CH bioaerosols have a greater prevalence of bacteria,

some of which have been shown to cause respiratory dysfunction. Therefore, bacterial diversity may help to explain the greater prevalence of respiratory symptoms in workers from CH operations. Also, area and personal DGGE profiles share less than 10% similarity, regardless of barn type. These data suggest that area samples may not accurately represent personal exposures and will be useful for better choice of sampling method in future studies.

2.7 Acknowledgements

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Data from chapter two "Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations" revealed that personal levels of dust, endotoxin, and bacteria were significantly higher in FH bioaerosols than CH bioaerosols but that biodiversity profiles from CH and FH personal bioaerosols shared less than 20% similarity. These results suggest that bacterial diversity may help to explain the higher prevalence of respiratory symptoms in CH workers but that total personal dust, endotoxin or bacteria concentrations may not explain the higher prevalence of respiratory symptoms in CH workers. Poultry bioaerosols are heterogeneous and it is possible that the respiratory symptoms observed in workers are caused by unknown exposures. Archaea have been detected in swine [29] and dairy [28] bioaerosols but poultry bioaerosols have never been examined for the presence of archaea. Chapter three "Archaeal characterization of bioaerosols from cage-housed and floor-housed poultry operations" examines levels of methanogenic archaea, another possible bioaerosol contaminant, in bioaerosols from CH and FH poultry operations.

3.0 CHAPTER THREE: Archaeal characterization of bioaerosols from cage-housed and floor-housed poultry operations

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Author Contributions

All authors participated in the design of the experiments and contributed to writing of the manuscript. Blais Lecours was responsible for qPCR for archaea and PCR-DGGE was performed by Marcoux-Voiselle. The author of this thesis contributed to sample collection, dust measurement, DNA extraction, qPCR for bacteria, and statistical analysis.

3.1 Abstract

Background. Although bioaerosols from both cage-housed (CH) and floor-housed (FH) poultry operations are highly concentrated, dust, endotoxin, and bacteria concentrations are significantly higher in FH bioaerosols. Workers from CH operations have reported a greater prevalence of respiratory symptoms. Archaea have been examined in swine and dairy bioaerosols but not in poultry bioaerosols. *Objective.* The objective of this study was to directly compare methanogenic archaea concentrations in bioaerosols from CH and FH poultry facilities. *Methods.* Bioaerosols were collected from fifteen CH and fifteen FH poultry operations, using stationary area samplers as well as personal sampling devices. Archaea were quantified and their diversity was investigated using PCR followed by denaturing gradient gel electrophoresis (DGGE) and band sequencing. *Results.* Archaea were significantly higher in area and personal bioaerosols from CH poultry operations than FH bioaerosols ($p<0.001$ and $p<0.05$, respectively) and did not differ significantly between area and personal samples within each barn type. Sequences matching *Methanobrevibacter woesei*, an archaeon previously found in poultry samples, were detected in bioaerosol samples from CH operations. *Conclusions.* Archaea concentrations are significantly different between bioaerosols from CH and FH poultry operations.

3.2 Introduction

Poultry barn environments contain high levels of dust, gases and odours [7]. Compared to non-farming controls, poultry workers report a higher prevalence of work-related eye, respiratory and skin symptoms [51]. They also report a higher prevalence of

chronic bronchitis, organic dust toxic syndrome (ODTS), and work-related lower and upper respiratory symptoms, including cough, phlegm, dyspnea, eye irritation, fatigue, headache, nasal congestion, fever, throat irritation, chest tightness and wheeze, than other agricultural workers [52, 78].

Levels of environmental contaminants in poultry dust vary depending on the type of housing. There are two common types of poultry housing facilities: cage-housed (CH) or layer operations, where birds are housed in cages for egg production, and floor-housed (FH) or broiler operations, where birds are housed on the floor for meat production. Differences between the two types of poultry operations include: worker time spent in direct contact with birds, predominance of female poultry in CH facilities, presence of eggs in CH operations, presence and type of litter in FH facilities, age of birds, length of time birds spend in housing and housing management practices [3]. Greater current and chronic phlegm have been reported in workers from CH facilities, despite dust, endotoxin and bacteria levels being significantly higher in FH operations [1, 63, 77, 95].

Archaea are similar in size and shape to bacteria [22], have Bacteria-like metabolism, and no nucleus or organelles [23]. However, Archaea have genetic, transcriptional and translational pathways similar to Eukaryotes [22, 23]. Archaea were named as such because they were originally detected in extreme environments, the most primitive locations on earth [22]. However, they are also found in non-extreme environments, digestive tracts of many animals [23, 24], and intestinal, vaginal and oral mucosa in humans [22]. Gastrointestinal archaea use various organic substrates, including alcohols, organic acids, CO_2 and H_2, to produce methane [25].

Methanogenic archaea have been detected in broiler fecal samples [26] and ceca of layer hens [27]. To date, airborne archaea have been detected in swine and dairy bioaerosols [28, 29] but have never been examined in bioaerosols from poultry operations. It is hypothesized that archaea will be present in poultry bioaerosols. The response following exposure to airborne archaea is poorly understood. Recent results show that two methanogens, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, induce airway inflammatory responses following intranasal exposure in mice [30]. Therefore, it is possible that airborne archaea may play a role in poultry worker respiratory health outcomes. This is the first study to focus on comparing archaea concentrations between bioaerosols from CH and FH poultry operations.

3.3 Materials and methods

3.3.1 Bioaerosol sampling

Air sampling was performed by Kirychuk *et al.* at 15 cage-housed (CH) and 15 floor-housed (FH) poultry operations in Saskatchewan, described previously [77]. Briefly, two area (A) samples and one personal (P) sample were collected at each barn. Dust was collected on pre-weighed radial slit polyvinyl chloride (PVC) filters (5μm) using a Marple cascade impactor (Thermo Electron Corp., Waltham, MA, USA) connected to a constant airflow pump (Universal 224-PCXR4; SKC, Eighty Four, PA, USA) run at 2L/min over a 4h sampling time. Six stages (3 through 8, with cut-points 0.52, 0.93, 1.55, 3.5, 6.0 and 9.8μm) were included in the Marple sampler. Only results from dust fractions >3.5μm (stages 3, 4 and 5) are reported here.

3.3.2 Dust and endotoxin analysis

Dust and endotoxin were measured by Kirychuk *et al.*, described previously [77]. Briefly, gravimetric analyses were performed to measure dust (MX5 microbalance; Mettler-Toledo, Greifensee, Switzerland). Dust from individual filters was extracted in 10mL sterile, pyrogen-free, endotoxin-free water (LAL reagent water; BioWittaker, Walkersville, MD, USA) and rocked at room temperature for 60min (Labquake shaker; Labindustries, Berkeley, CA, USA). Aliquots of 0.5mL were applied to kinetic-QCL Limulus Amoebocyte Lysate (LAL) assays to quantify endotoxin (*Escherichia coli* O55: B5; Cambrex BioScience Walkersville Inc, Walkersville, MD). Dust concentrations were expressed as milligrams per cubic meter of air (mg m^{-3}) and endotoxin concentrations were expressed as endotoxin units per cubic meter of air (EU m^{-3}).

3.3.3 DNA extraction

Aliquots of 1.5mL extracted dust were centrifuged (10min, 21000g, room temperature) and pellets were stored at -20°C until DNA extraction. Isolation of total genomic DNA was performed using the QIAamp DNA Mini Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions for tissue with modifications for bacteria. Total DNA samples were eluted in 100μL elution buffer, supplied with the kit.

3.3.4 Quantitative real-time PCR for bacteria

Amplification was performed using a DNA Engine Opticon2 (Bio-Rad, Mississauga, ON, Canada) and all primers and DNA probes (Table I) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Bacteria quantification was

performed as described previously [95] with 16S rRNA forward primer (5'-GGTAGTCYAYGCMSTAAACG-3'), 16S rRNA reverse primer (5'-GACARCCATGCASCACCTG-3') and 16S rRNA probe (FAM-TKCGCGTTGCDTCGAATTAAWCCAC-IB^{TM}FQ) [85] (Table I). The PCR components were as follows: 2.5μL DNA template, 0.4μM each primer, 0.08μM probe, 0.5U uracil N-glycosylase (UDG) (Sigma, Oakville, ON, Canada) and 12.5μL 2X QuantiTect Probe PCR kit (Qiagen, Mississauga, ON, Canada) in a 25μL reaction. The PCR program was as follows: hold at 37°C for 10min, hold at 95°C for 15min then 40 cycles of 95°C for 20s and 62°C for 60s. Ten-fold serial dilutions of *E. coli* genomic DNA were used for the standard curve (efficiency=99.96%, r^2=0.991). Data were collected with the Opticon Monitor software 2.02.24. The absolute 16S rRNA copy number was multiplied by 1.75, the ratio of 16S rRNA copies in *E. coli* (7) to the average of all bacteria (4), to determine the number of bacteria in each sample. Field blanks and negative controls were included to detect PCR reagent contamination.

3.3.5 Quantitative real-time PCR for archaea

Amplification was performed using a DNA Engine Opticon2 (Bio-Rad, Mississauga, ON, Canada) and all primers (Table I) were purchased from Integrated DNA Technologies (Coralville, IA, USA). Archaea quantification was performed as described previously [28] with 16S rRNA forward primer A751F (5'-CCGACGGTGAGRGRYGAA-3') [96] and 16S rRNA reverse primer A976R (5'-YCCGGCGTTGAMTCCAATT-3') [97] (Table I). The PCR components were as follows: 2μL DNA template, 0.5μM each primer, and 12.5μL iQ^{TM} SYBR Green

Supermix (Bio-Rad Laboratories, Hercules, USA) in a 25μL reaction. The PCR program was as follows: hold at 94°C for 5min then 35 cycles of 94°C for 10s, 55.5°C for 20s, plate read and 72°C for 25s, followed by 10min at 72°C. The following melting curve program was performed: 40°C to 94°C, read every 0.2s, hold 1s. Samples were considered positive for archaeal 16S rRNA genes when the melting temperature was around 88°C. Ten-fold serial dilutions of *Methanosarcina mazei* genomic DNA (ATCC BAA-159D) were used for the standard curve (efficiency=86.21%, r^2=0.997). Data were collected with the Opticon Monitor software 2.02.24 and threshold was determined with a standard deviation = 1. The absolute 16S rRNA copy number was multiplied by 1.775, the ratio of 16S rRNA copies in *M. mazei* (3) to the average of all archaea (1.69), to determine the number of archaea in each sample. Field blanks and negative controls were included to detect PCR reagent contamination.

Table I. Primers

Primer	Target	Sequence	Reference
EUBf	Bacteria 16S rRNA	GGTAGTCYAYGCMSTAAACGT	Bach *et al*. 2002
EUBr	Bacteria 16S rRNA	GACARCCATGCASCACCTG	Bach *et al*. 2002
EUBp	Bacteria 16S rRNA	FAM-TKCGCGTTGCDTCGAATTAAWCCAC-IB^{TM}FQ	Bach *et al*. 2002
A751F	Archaea 16S rRNA	CCG ACG GTG AGR GRY GAA	Baker *et al.*, 2003
A976R	Archaea 16S rRNA	YCC GGC GTT GAM TCC AAT T	Reysenbach and Pace, 1995
A333F	Archaea 16S rRNA	TCC AGG CCC TAC GGG	Reysenbach and Pace, 1995
A751R*	Archaea 16S rRNA	TTC RYC YCT CAC CGT CG	Baker *et al.*, 2003

All primers were purchased from IDT (Coralville, IA, USA)

* GC-clamp,CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C (Muyzer *et al.*, 1993)

3.3.6 Denaturing gradient gel electrophoresis (DGGE) analysis

DNA from stages 3, 4 and 5 were pooled, as well as both area samples, prior to DGGE analysis. Archaea concentrations from FH bioaerosols were too low for DGGE and sequencing analyses. Therefore, 30 samples (15 CH area and 15 CH personal) were

characterized. The variable regions V4 and V5 of the 16S rRNA gene from nucleotide 333 to 751 were amplified using primers A333F (5'-TCCAGGCCCTACGGG-3') [97] and A751R (5'-TTCRYCYCTCACCGTCG-3') [96] including a GC clamp [80] (Table I) as described previously [28]. The PCR components were as follows: 2μL DNA template, 0.5μM each primer, 3.5mM $MgCl_2$, 100μM dNTP, 5% v/v DMSO, and 2.5U *Taq* (Promega) polymerase in a 50μL reaction. The PCR program was as follows: 94°C for 45s then 35 cycles of 94°C for 30s, 54.8°C for 65s, 72°C for 60s, followed by 10min at 72°C. PCR was performed by the DNA Engine DYAD™ thermocycler (Bio-Rad, Mississauga, ON, Canada). Following 1% agarose gel electrophoresis, DNA was quantified by comparing band intensities to the BioRad molecular mass ladder (Bio-Rad, Mississauga, ON, Canada) measured with GeneTools software (SynGen, Cambridge, England). Denaturing gradient gel electrophoresis (DGGE) was performed as described previously [28] using the DCode (Bio-Rad, Mississauga, ON, Canada). PCR products (30ng) were loaded on 8% polyacrylamide gels in 1X TAE buffer (Bio-Rad, Mississauga, ON, Canada) with a 25-65% denaturing gradient (100% denaturant was 7M urea and 40% v/v deionized formamide). Electrophoresis was performed at 60V for 16h at 60°C. Gels were stained for 15min in 0.5X TAE with SYBR Gold (Molecular Probes, Eugene, OR, USA) and destained twice for 15min each. Gel images were obtained with the imaging system ChemiGenius 2 and GeneSnap software (SynGen, Cambridge, England).

3.3.7 Sequencing analysis

DNA was excised from bands in the gels using micropipette tips, which were placed in tubes containing aliquots of PCR reagents for 5min prior to re-amplification.

The PCR products were visualized by agarose gel electrophoresis and sequenced on both strands (CHUL Research Center, Québec, QC, Canada). Each DNA sequence was compared to the Genbank database from the National Center of Biotechnology Information using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were checked for chimeras using DECIPHER's Find Chimeras web tool (http://DECIPHER.cee.wisc.edu/FindChimeras.html).

3.3.8 Statistical analysis

Archaea concentrations are reported as raw data. However, log transformation of the data was required prior to statistical analyses. The following comparisons were made using unpaired Student's *t*-tests: FH area vs. CH area, FH personal vs. CH personal, FH area vs. FH personal and CH area vs. CH personal. Results with p-values < 0.05 were considered significant.

3.4 Results

3.4.1 Archaea concentrations

Archaea were measured using quantitative real-time PCR targeting the 16S rRNA gene. Archaea were detected in 7/15 (46.7%) FH area bioaerosols and 15/15 (100%) CH area bioaerosols, 8/15 (53.3%) FH personal bioaerosols and 12/15 (80.0%) CH personal bioaerosols. Average archaea concentrations for FH and CH area bioaerosols were 1.2×10^4 and 1.2×10^6 archaea m^{-3}, respectively. Average archaea concentrations for FH and CH personal bioaerosols were 2.6×10^4 and 6.5×10^5 archaea m^{-3}, respectively. Area and personal archaea concentrations were significantly higher in bioaerosols from CH

poultry operations than FH bioaerosols ($p<0.001$ and $p<0.05$) (Figure 1). No significant difference in archaea concentration was observed between area and personal bioaerosols, within each barn type (FH: $p=0.55$, CH: $p=0.25$).

Dust, endotoxin and bacteria concentrations were higher in FH area and personal bioaerosols than those from CH operations, while archaea concentrations are higher in CH bioaerosols (Figure 2). In bioaerosols from FH facilities average bacteria concentrations were higher than average archaea whereas in CH bioaerosols average archaea concentrations were higher than average bacteria (Figure 2).

3.4.2 Sequencing analysis

Archaeal species within poultry bioaerosols were identified using DGGE analysis followed by sequencing. Only amplification of archaeal DNA from CH bioaerosol samples for DGGE and sequencing analyses was possible as concentrations were too low in FH bioaerosol samples. Sequences matching the following archaeal affiliations were detected in CH bioaerosols: *Methanobrevibacter woesei*, *Methanosarcina mazei* and *Haloquadratum walsbyi* (Table II). All sequences had high similarity (100%) with affiliated archaea. Bands present in multiple DGGE profiles were sequenced to confirm their affiliation to the same microorganism. Sequences matching *Methanobrevibacter woesei* were detected in CH bioaerosols from six area and three personal samples. *Methanosarcina mazei* sequence affiliations were detected in CH bioaerosols from one area and two personal samples. One sequence matching *Haloquadratum walsbyi* was detected in a single personal sample of CH bioaerosols.

Table II. Closest affiliations to sequences obtained from DGGE profiles of area and personal bioaerosols from cage-housed poultry operations

DGGE band affiliation (accession number)	**bp**	**% similarity**
Methanobrevibacter woesei (DQ445724.1)	321	100
Methanosarcina mazei (JN413085.1)	321	100
Haloquadratum walsbyi (FR746099.1)	217	100

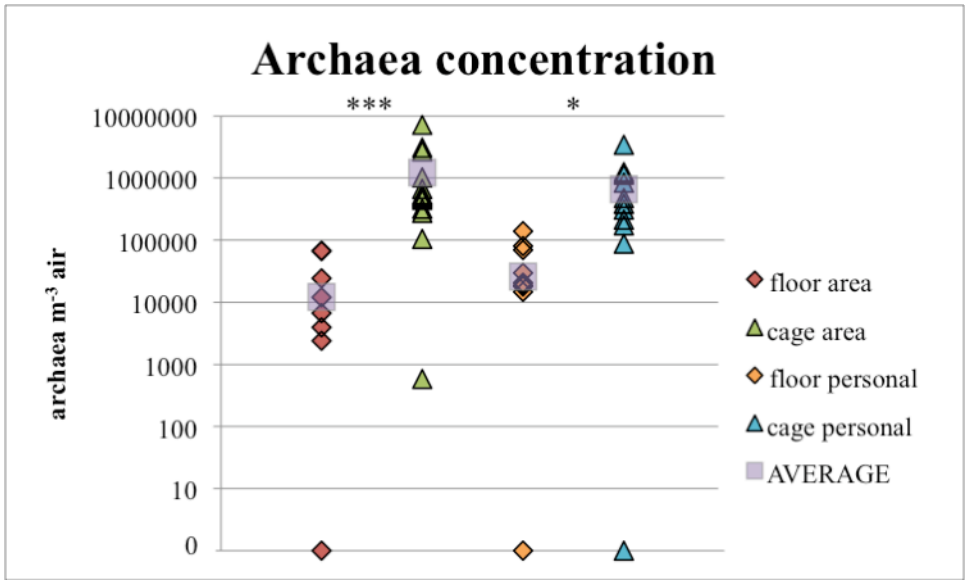
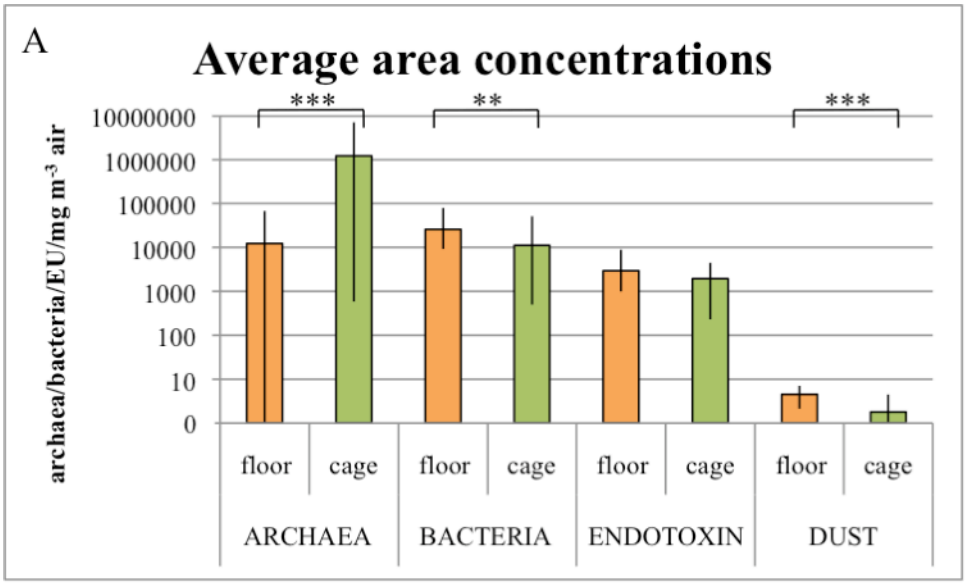


Figure 1. Quantification of airborne archaea by real-time PCR in cage-housed (CH) and floor-housed (FH) bioaerosols collected using area (A) and personal (P) sampling devices. Each point on the graph represents raw data from a single barn, averages indicated. Points along the x-axis represent barns where archaea were undetected in bioaerosols. Data were log transformed prior to statistical analyses. *$p<0.05$ ***$p<0.001$



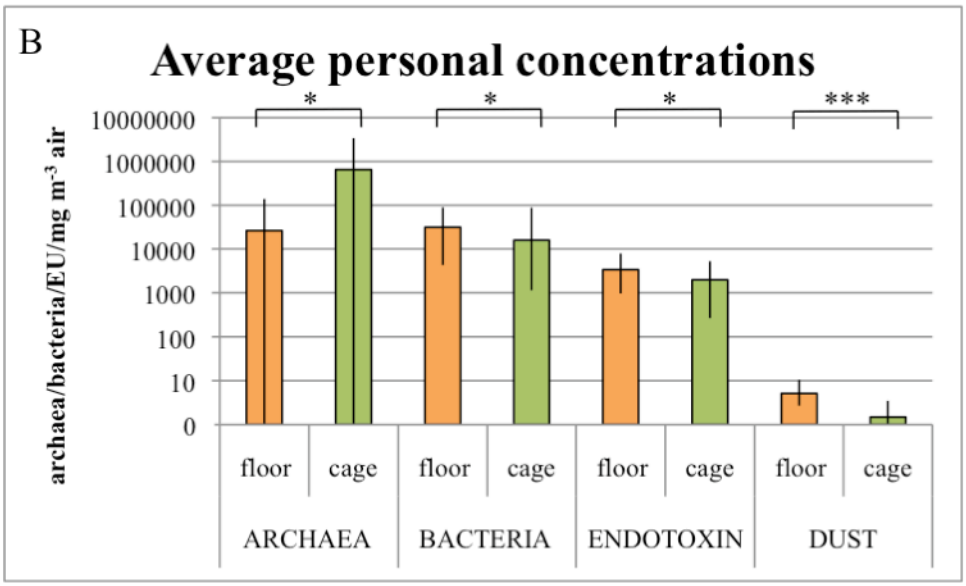


Figure 2. A) Area concentrations. B) Personal concentrations. Average archaea (archaea m^{-3} air), bacteria (bacteria m^{-3} air), endotoxin (EU m^{-3} air) and dust (mg m^{-3} air) concentrations in bioaerosols from cage-housed and floor-housed operations are reported. Data were log transformed prior to statistical analyses. *$p<0.05$ **$p<0.01$ ***$p<0.001$ Average dust, endotoxin and bacteria concentrations were published previously [95].

3.5 Discussion

We detected airborne archaea concentrations ranging from lower than the limit of detection to 7.2 x 10^6 archaea m^{-3} air (0.0 to 6.86 $\log_{10}$ archaea m^{-3} air). To date, there are no values of typical archaea concentrations in poultry bioaerosols. The concentrations that we detected are between the reported 10^4 to 10^6 16S rRNA genes m^{-3} air in dairy barns [28] and the reported 10^6 to 10^8 archaea m^{-3} air in swine confinement buildings [29]. Total archaea concentrations correlated with total bacteria concentrations in swine bioaerosols [29] but not in dairy bioaerosols [28]. We observed a trend where archaea concentrations were higher in bioaerosols from CH facilities that had higher bacteria concentrations. This trend was not observed in bioaerosols from FH operations and may be explained by the higher number of negative samples from FH facilities than CH operations.

It was hypothesized that dust, endotoxin, bacteria and/or archaea would differ between CH bioaerosols and FH bioaerosols due to the different management practices between these two barn types and the greater prevalence of respiratory symptoms in workers from CH facilities [1]. Bacterial diversity profiles from CH and FH personal bioaerosols shared less than 20% similarity [95]. In contrast to dust, endotoxin, and bacteria concentrations, which were higher in bioaerosols from FH operations [77, 95], archaea concentrations are significantly higher in bioaerosols from CH facilities compared to FH operations. Our results are supported by previously published studies of archaea from layer ceca and broiler fecal poultry samples. Methanogenic archaea concentrations ranged from 4.19 to 5.34 $\log_{10}$ 16S rRNA copies g^{-1} (wet weight) in broiler fecal samples [26] and 5.50 to 7.19 $\log_{10}$ 16S rRNA copies g^{-1} (wet weight) in

layer ceca samples [27]. Of the 11 sequences detected in broiler ceca samples, 10 shared 98.97-99.45% similarity to *Methanobrevibacter woesei* [27]. Miller *et al.* first detected *M. woesei* in goose feces [27, 98, 99]. In our study, 9/13 (69.2%) sequences detected from CH bioaerosols matched *Methanobrevibacter woesei*, supporting the observation that *M. woesei* is the predominant methanogen in chicken ceca [27]. Fewer bands were detected in DGGE profiles from poultry bioaerosols than swine [29] and dairy bioaerosols [28], supporting the claim that nonruminant animals have less archaeal diversity than ruminants [27].

The possible role of archaea in airway inflammation and respiratory symptoms is poorly understood. Two methanogenic archaeal species, *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*, induce airway inflammatory responses following intranasal exposure in mice [30]. It will be necessary to investigate the response following airway exposure to *Methanobrevibacter woesei*. Greater knowledge of bioaerosol content in various occupational settings is essential in order to better understand the potential health effects of exposed humans.

Just as bacteria acquire antimicrobial resistance, archaea can become resistant to antimicrobials. Archaea are susceptible to antimicrobials that are effective against bacteria and eukaryotes such as bacitracin, an antibiotic that targets the lipid cycle and is commonly used in the poultry industry [22, 100]. It is possible that the use of antibiotics in FH facilities may play a role in the low concentrations of archaea detected in FH bioaerosols compared to CH bioaerosols. It is also possible that the bacterial zinc bacitracin resistance gene *bcr*R, which is more prevalent in FH bioaerosols [101], may be

acquired by archaea. Further investigation of antimicrobial resistance in archaea from poultry bioaerosols is required.

This study is limited by the methodology of sequence identification. DGGE followed by sequencing uses a relatively short DNA sequence, which could result in less precise sequence identification. Also, the high number of PCR cycles required prior to DGGE is known to decrease sequence diversity [92]. Amplification of different variable regions of the 16S rRNA gene followed by denaturing gradient gel electrophoresis results in different profiles [102] and closely related organisms may have nearly identical sequences [103]. Due to the low sequence divergence of the 16S rRNA gene, sequences sharing less than 98% similarity represent different species of archaea [98]. The affiliated sequences detected in this study are not exhaustive and relevant archaea may be overlooked. This study provides qualitative data of archaeal diversity and can only draw conclusions on archaea concentration.

This is the first known examination of archaeal quantity and diversity in poultry bioaerosols. It is also the first comparison of airborne archaea between CH and FH poultry operations. Little information exists on either airborne archaea or comparisons of the two types of poultry operations so this work is an important contribution.

3.6 Conclusions

Biological contaminants are high in poultry bioaerosols and poultry workers report respiratory dysfunction. Management practices differ between CH and FH poultry operations and CH workers report a greater prevalence of respiratory symptoms [1]. Therefore, it is expected that levels and types of biological contaminants may differ

between these two barn environments. Dust, endotoxin and bacteria concentrations were higher in FH bioaerosols than CH bioaerosols [95]. Archaea concentrations are significantly greater in bioaerosols from CH operations than FH facilities and sequences matching *Methanobrevibacter woesei* are detected in CH bioaerosols.

3.7 Acknowledgements

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Data from chapter two "Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations" revealed that biodiversity profiles from CH and FH personal bioaerosols shared less than 20% similarity, that biodiversity profiles from barns where antibiotics were used clustered together, and that sequences matching bacteria known to harbour antimicrobial resistance genes (ARGs), such as *Staphylococcus cohnii*, are detected in bioaerosols from CH and FH poultry barns. These results suggest that bacterial diversity may help to explain the higher prevalence of respiratory symptoms in CH workers, that antibiotic use may influence biodiversity, and that antimicrobial resistant bacteria may be present in poultry bioaerosols. Data from chapter three "Archaeal characterization of bioaerosols from cage-housed and floor-housed poultry operations" revealed that methanogenic archaea concentrations were significantly greater in CH bioaerosols than FH bioaerosols. Archaea are intrinsically sensitive to zinc bacitracin, an antibiotic that is commonly used in the poultry industry. Archaea may develop antimicrobial resistance as bacteria do. Chapter four "Potentially pathogenic bacteria and antimicrobial resistance in bioaerosols from cage-housed and floor-housed poultry operations" detects specific bacterial species and ARGs in bioaerosols from CH and FH poultry operations.

4.0 CHAPTER FOUR: Potentially pathogenic bacteria and antimicrobial resistance in bioaerosols from cage-housed and floor-housed poultry operations

Ann Occup Hyg. 2012 May;56(4):440-9.

Just, N., Létourneau, V., Kirychuk, S., Singh, B. and Duchaine, C. (2012). *Potentially pathogenic bacteria and antimicrobial resistance in bioaerosols from cage-housed and floor-housed poultry operations.* Annals of Occupational Hygiene. **56**(4): p. 440-9.

Author Contributions

All authors participated in the design of the experiments and contributed to writing of the manuscript. Létourneau performed end-point PCR. The author of this thesis contributed to sample collection, dust measurement, DNA extraction, qPCR, and statistical analysis.

4.1 Abstract

Background. Antibiotics are used in animal confinement buildings, such as cage-housed (CH) and floor-housed (FH) poultry operations, to lower the likeliness of disease transmission. In FH facilities, antibiotics may also be used at sub-therapeutic levels for growth promotion. Low levels of antibiotic create a selective pressure towards antimicrobial resistance (AMR) in chicken fecal bacteria. *Objective.* The objective of this study was to compare bacteria and AMR genes in bioaerosols from CH and FH poultry facilities. *Methods.* Bioaerosols were collected from fifteen CH and fifteen FH poultry operations, using stationary area samplers as well as personal sampling devices. Bacteria concentrations were determined by genus or species specific quantitative PCR and AMR genes were detected using end-point PCR. *Results.* *Enterococcus* spp., *E. coli* and *Staphylococcus* spp. were significantly higher in bioaerosols from FH poultry operations than CH bioaerosols ($p<0.001$) while *C. perfringens* was significantly higher in area bioaerosols from CH operations than FH area bioaerosols ($p<0.05$). *Campylobacter* spp. were detected only in bioaerosols from FH facilities. Zinc bacitracin resistance gene, *bcr*R, erythromycin resistance gene, *erm*A, and tetracycline resistance gene, *tet*A/C, were more prevalent in bioaerosols from FH facilities than CH bioaerosols ($p<0.01$, $p<0.01$ and $p<0.05$, respectively). *Conclusions.* Most bacteria are more concentrated and most AMR genes are more prevalent in bioaerosols from FH poultry operations, where growth-promoting antibiotics are used.

4.2 Introduction

Poultry facilities are associated with high production of dust, gases and odours [7]. In poultry bioaerosols, bacteria exist suspended freely in the air as well as attached to dust particles [7]. Bacteria commonly found in the poultry industry include *Campylobacter* spp., *Staphylococcus* spp., *Salmonella* spp., *Clostridium perfringens* type A, *Enterococcus faecalis* and *Escherichia coli* [4, 15, 32, 35, 37, 95, 104-106]. The type of housing may influence levels of environmental contaminants in poultry dust. Within the poultry production industry, there are two common types of facilities: cage-housed (CH) operations, where birds are housed in cages for egg production, and floor-housed (FH) operations, where birds are housed on the floor for meat production. There are a number of differences in the two types of poultry operations including: time workers spend in direct contact with birds, predominance of female poultry in CH facilities, presence of eggs in CH operations, presence and type of litter in FH facilities, age of birds, length of time birds spend in housing and housing management practices [3].

Over the past few decades, Canadian poultry production has shown a trend towards housing a greater number of birds per operation. This change has increased air contamination and the need for antibiotics, as the closer proximity of animals increases the likeliness of disease transmission [107]. Antibiotics are also used at sub-therapeutic levels in feed or water for growth promotion. Sub-therapeutic antibiotic levels create a selective pressure towards antimicrobial resistance (AMR) in chicken fecal bacteria [108]. Antibiotics such as gentamicin, neomycin, erythromycin, penicillin, virginiamycin, tetracyclines and zinc bacitracin are commonly used for growth promotion or prophylaxis during poultry production [35, 36].

Many of the bacteria found in poultry environments are known to cause infection and/or disease in both animals and humans and most of the antibiotics used in poultry production are also used in human medicine. Therefore, workers may be exposed to antibiotics and potential pathogens, promoting antimicrobial resistant infections [41, 108, 109]. There is also a risk that workers are a potential reservoir of antibiotic resistant bacteria [110]. Transmission of antimicrobial resistant bacteria can occur through direct contact, contaminated water, air, environment and food [41]. Although airborne transmission of bacteria among animals has been reported in the poultry and swine industries [43, 44], respiratory transmission to agricultural workers is poorly characterized in the literature. One case study suggested that a poultry worker acquired *Campylobacter* through orally transmitted water droplets [111].

Recently, we have shown differences in total bacteria concentrations and bacterial diversity between CH and FH bioaerosols [95]. Antibiotic use for growth promotion is limited to FH poultry operations. These data suggest that exposure to antimicrobial resistant bacteria may differ between CH and FH workers. This study investigates the presence of potentially pathogenic bacteria and antimicrobial resistant genes in bioaerosols from CH and FH poultry operations.

4.3 Materials and methods

4.3.1 Bioaerosol sampling

Air sampling was performed by Kirychuk *et al.* at 15 cage-housed (CH) and 15 floor-housed (FH) poultry operations in Saskatchewan, described previously [77]. Briefly, two area (A) samples and one personal (P) sample were collected at each barn.

Dust was collected on pre-weighed radial slit polyvinyl chloride (PVC) filters using a Marple cascade impactor (5μm; Thermo Electron Corp., Waltham, MA, USA) connected to a constant airflow pump (Universal 224-PCXR4; SKC, Eighty Four, PA, USA) run at 2L/min over a 4h sampling time. Six stages (3 through 8, with cut-points 0.52, 0.93, 1.55, 3.5, 6.0 and 9.8μm) were included in the Marple sampler. Only results from dust fractions >3.5μm (stages 3, 4 and 5) are reported here, as bacteria were detected in only these fractions. None of the barns visited reported recent disease outbreaks.

4.3.2 DNA extraction

DNA extraction was performed as described previously [95]. Dust from individual filters was extracted in 10mL sterile, pyrogen-free, endotoxin-free water (LAL reagent water; BioWittaker, Walkersville, MD, USA) and rocked at room temperature for 60min (Labquake shaker; Labindustries, Berkeley, CA, USA). Aliquots of 1.5mL extracted dust were centrifuged (10min, 21000g, room temperature) and pellets were stored at -20°C until DNA extraction. Isolation of total genomic bacterial DNA was performed using the QIAamp DNA Mini Kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions for tissue with modifications for bacteria. Total DNA samples were eluted in 100μL elution buffer, supplied with the kit. DNA from stages 3, 4 and 5 were pooled, as well as both area samples, prior to PCR analysis. Therefore, 60 samples (15 CH area, 15 CH personal, 15 FH area and 15 FH personal) were characterized.

*4.3.3 Quantitative real-time PCR for bacteria and tet*G

Quantification of *Campylobacter*, *C. perfringens*, *Enterococcus*, *E. coli*, *Staphylococcus* and *tet*G was performed as described previously [110, 112-115]. Amplification was performed using a DNA Engine Opticon2 (Bio-Rad, Mississauga, ON, Canada) in duplicate, using SYBR Green I-based PCR. The PCR components per 25µl were as follows: 5µl template DNA, 0.25 µM of each primer and 12.5 µl 2X QuantiTect SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Kit, QIAGEN, Mississauga, ON, Canada). Target genes, primers, hybridization temperatures and amplicon lengths are indicated in Table I (Malinen *et al.*, 2003; Oppliger *et al.*, 2008; Rinttila *et al.*, 2004; Wise and Siragusa, 2005; Yu *et al.*, 2005;). Primers for *E. coli* target the *Escherichia* subgroup, composed of *E. coli*, *Hafnia alvei* and *Shigella* spp. (Malinen et al. 2003). All primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). The PCR program was as follows: one hold at 95^{o}C for 15min for DNA denaturation and activation of DNA polymerase followed by 40 cycles of 94^{o}C for 15s, hybridization for 30s (see Table I) and 72^{o}C for 30s. Fluorescence data were collected at the hybridization step (endpoint) with the Opticon Monitor software 2.02.24 and analyzed by linear regression $\log_{10}$(copy number)=f(threshold cycle). Following quantitative PCR, the presence of primer dimers and the specificity of target sequences were evaluated by melting curve analysis. Tenfold serial dilution of a DNA plasmid construct in sterile DNase-free water (10^6 to 10^0) was used as a standard curve and prepared prior to each PCR assay. The DNA plasmid construct consisted of pCR4TOPO with a pathogen-specific gene cloned in *E. coli* (TOPO TA cloning kit, Invitrogen, Burlington, ON). DNA samples were quantified against the standard. Bacterial

concentrations were expressed as copies of genes per cubic meter of air (genes/m^3). PCR efficiency was determined by $E=10^{(-slope)}-1$. The amplification efficiencies of our qPCR protocols were between 90 and 104% (r^2=0.992 to 0.999). The limits of detection for our protocol were 2 genes per reaction for *C. perfringens*, *Enterococcus*, *E. coli* and *tet*G, and 20 genes per reaction for *Campylobacter* and *Staphylococcus*. Negative controls were included to detect PCR reagent contamination.

Table I. Target genes, primers, hybridization temperatures, amplicon lengths and references for PCR.

Target gene	Sequence	Hybridization temperature	Amplicon length	Reference
16S rRNA (*Campylobacter*)	GGATGACACTTTTCGGAG AATTCCATCTGCCTCTCC	58°C	246 bp	Rinttila *et al.* 2004
16S rRNA (*C. perfringens*)	CGCATAACGTTGAAAGATGG CCTTGGTAGGCCGTTACCC	57°C	105 bp	Wise and Siragusa 2005
16S rRNA (*Enterococcus*)	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	57°C	144 bp	Rinttila *et al.* 2004
16S rRNA (*E. coli*)	GTTAATACCTTTGCTCATTGA ACCAGGGTATCTAATCCTGTT	55°C	340 bp	Malinen *et al.* 2003
tuf (*Staphylococcus*)	GGCCGTGTTGAACGTGGTCAAATCA TIACCATTTCAGTACCTTCTGGTAA	55°C	370 bp	Oppliger *et al.* 2008
*bcr*R	GTTACCCTAACATGGAGTCG AAACATAACCGCCAACAGAG	55°C	215 bp	Thibodeau *et al.* 2008
*erm*A	GAAATYGGRTCAGGAAAAGG AAYAGYAAACCYAAAGCTC	55°C	332 bp	Chen *et al.* 2007
*erm*B	GATACCGTTTACGAAATTGG GAATCGAGACTTGAGTGTGC	58°C	364 bp	Chen *et al.* 2007
*tet*A/C	GCTRTATGCGTTGTGCAAT TCCTCGCCGAAAATGACC	58°C	567 bp	Yu *et al.* 2005
*tet*G	GTCGATTACACGATTATGGC CACTTGGCCGATCAGTTGA	57°C	432 bp	Yu *et al.* 2005

All primers were purchased from Integrated DNA Technologies (Coralville, IA, USA).

*4.3.4 End-point PCR for AMR genes bcr***R***, erm***A***, erm***B** *and tet***A/C**

Detection of *tet*A/C was performed as described by Létourneau *et al.* [110, 113]. Detection of *erm*A and *erm*B was performed as described by Chen *et al.* [116]. Detection

of *bcr*R was performed as described by Thibodeau *et al.* [36]. Control strains for *erm*B and *bcr*R were kindly provided by Dr. Ann Letellier (Faculty of Veterinary Medicine, University of Montreal). Primers, hybridization temperatures and amplicon lengths are indicated in Table I. Negative controls were included to detect PCR reagent contamination. Barns were reported as positive if AMR genes were detected in both area and personal samples.

4.3.5 Statistical analysis

Bacteria and *tet*G concentrations are reported as raw data. However, log transformation of the data was required prior to statistical analyses. The following comparisons were made using unpaired Student's *t*-tests: FH area samples vs. CH area samples and FH personal samples vs. CH personal samples. Prevalence of bacteria and AMR genes in FH vs. CH operations was compared using Fisher's Exact test. Results with p-values < 0.05 were considered significant.

4.4 Results

4.4.1 Bacteria concentrations in area bioaerosols

Campylobacter, *C. perfringens*, *Enterococcus*, *E. coli* and *Staphylococcus* were measured by quantitative PCR in bioaerosols collected with area samplers in floor-housed (FH) and cage-housed (CH) poultry operations. Among FH operations, average bacteria concentrations were 1.3×10^7 *Staphylococcus tuf*/m^3, 3.8×10^5 *Enterococcus 16S rRNA*/m^3, 3.7×10^5 *E. coli 16S rRNA*/m^3, 7.2×10^4 *Campylobacter 16S rRNA*/m^3 and 1.5×10^4 *C. perfringens 16S rRNA*/m^3. Among CH facilities, average bacteria

concentrations were $9.2x10^4$ *Staphylococcus tuf*/m^3, $6.2x10^4$ *Enterococcus 16S rRNA*/m^3, $5.4x10^4$ *C. perfringens 16S rRNA*/m^3 and $1.2x10^4$ *E. coli 16S rRNA*/m^3. *Enterococcus*, *E. coli* and *Staphylococcus* were significantly more concentrated in FH area bioaerosols than CH area bioaerosols ($p<0.001$), while *C. perfringens* was significantly more concentrated in CH area bioaerosols compared to FH area bioaerosols ($p<0.05$) (Figure 1A).

4.4.2 Bacteria concentrations in personal bioaerosols

Campylobacter, *C. perfringens*, *Enterococcus*, *E. coli* and *Staphylococcus* were measured by quantitative PCR in bioaerosols collected with personal samplers in floor-housed (FH) and cage-housed (CH) poultry operations. Among FH operations, average bacteria concentrations were $1.2x10^7$ *Staphylococcus tuf*/m^3, $7.7x10^5$ *Enterococcus 16S rRNA*/m^3, $2.0x10^5$ *E. coli 16S rRNA*/m^3, $1.3x10^5$ *Campylobacter 16S rRNA*/m^3 and $3.8x10^4$ *C. perfringens 16S rRNA*/m^3. Among CH facilities, average bacteria concentrations were $3.2x10^5$ *Staphylococcus tuf*/m^3, $2.1x10^5$ *Enterococcus 16S rRNA*/m^3, $6.4x10^4$ *C. perfringens 16S rRNA*/m^3 and $1.6x10^4$ *E. coli 16S rRNA*/m^3. *Enterococcus*, *E. coli* and *Staphylococcus* were significantly more concentrated in FH personal vs. CH personal bioaerosols ($p<0.001$), while *C. perfringens* was not significantly different between CH personal and FH personal bioaerosols ($p=0.36$) (Figure 1B).

4.4.3 Bacteria prevalence

Barns were considered positive if the bacteria being investigated were detected in both area and personal bioaerosol samples. All 15/15 FH facilities were positive for

Enterococcus, *E. coli* and *Staphylococcus*. However, 14/15, 4/15 and 8/15 CH operations were positive for these organisms, respectively. *E. coli* and *Staphylococcus* were significantly more prevalent in FH poultry operations ($p<0.001$ and $p<0.01$, respectively). A single FH facility was positive for *Campylobacter*. *C. perfringens* was detected in 9/15 CH operations and 6/15 FH facilities (Figure 2).

4.4.4 Antimicrobial resistance concentrations and prevalence

Tetracycline resistance gene (*tet*G) was detected by quantitative PCR in area bioaerosols from 5/15 FH and 4/15 CH poultry operations, at an average of $6.9x10^3$ and $7.8x10^3$ genes/m^3, respectively ($p=0.54$) (Figure 1A). *tet*G was detected in personal bioaerosols from 5/15 FH and 5/15 CH poultry operations, at an average of $2.6x10^4$ and $1.8x10^4$ genes/m^3, respectively ($p=0.71$) (Figure 1B).

Zinc bacitracin resistance gene (*bcr*R), erythromycin resistance genes (*erm*A and *erm*B), and tetracycline resistance gene (*tet*A/C) were detected by endpoint PCR in bioaerosols from FH and CH poultry operations. Barns were considered positive if the AMR genes being investigated were detected in both area and personal bioaerosol samples. All barns were positive for at least one AMR gene, except for a single CH operation. Zinc bacitracin resistance gene (*bcr*R), erythromycin resistance gene (*erm*A), and tetracycline resistance gene (*tet*A/C) were detected at a higher prevalence in FH vs. CH facilities (*bcr*R: 15/15 FH vs. 8/15 CH $p<0.01$, *erm*A: 8/15 FH vs. 0/15 CH $p<0.01$, *tet*A/C: 13/15 FH vs. 6/15 CH $p<0.05$). Erythromycin resistance gene, *erm*B, was detected in 15/15 FH vs. 12/15 CH operations and tetracycline resistance gene, *tet*G, was detected in 3/15 FH and 3/15 CH facilities (Figure 3).

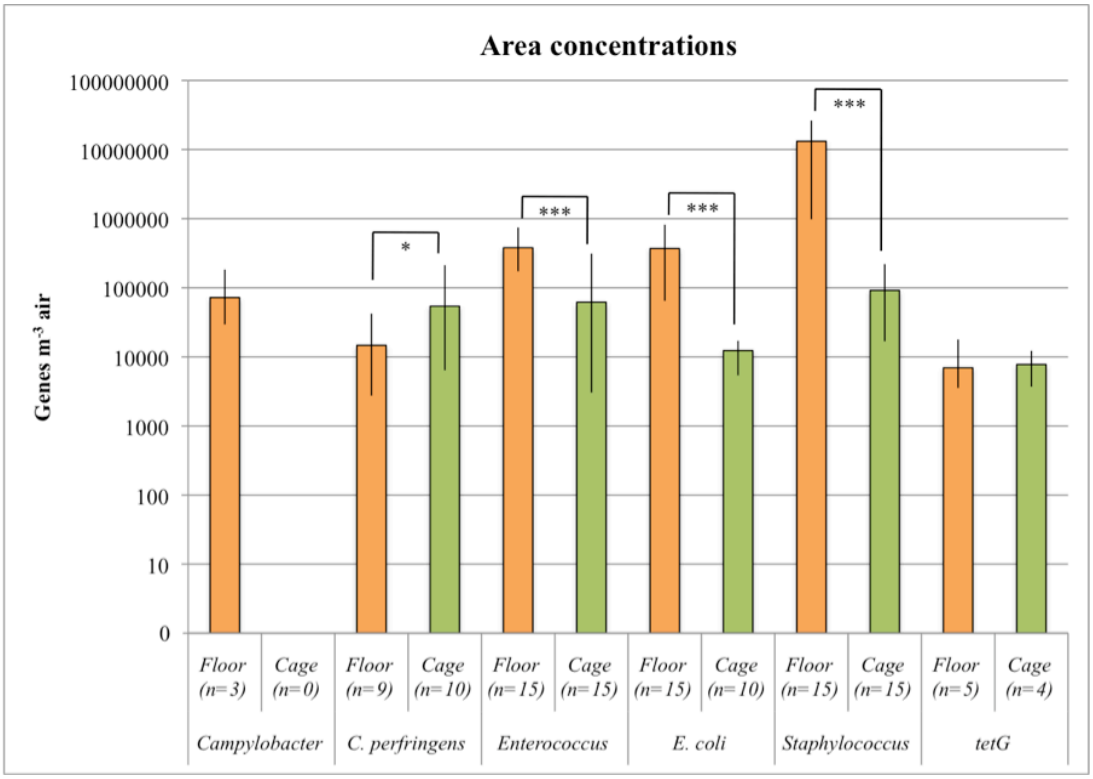

Figure 1A. Area concentrations. Bacteria and *tet*G concentrations were determined in bioaerosols from floor-housed (FH) and cage-housed (CH) poultry operations collected with area and personal samplers. Data is reported as genes per m^3 air (*16S rRNA* for *C. perfringens*, *Campylobacter*, *E. coli* and *Enterococcus*, *tuf* for *Staphylococcus* and *tet*G for tetracycline resistance). The number of positive samples is indicated (*n*). Target genes, primers, hybridization temperatures and amplicon lengths for quantitative PCR are indicated in Table I. Raw data, averages with ranges indicated, is presented in the graphs. Data was log transformed prior to statistical analyses. *$p<0.05$ ***$p<0.001$

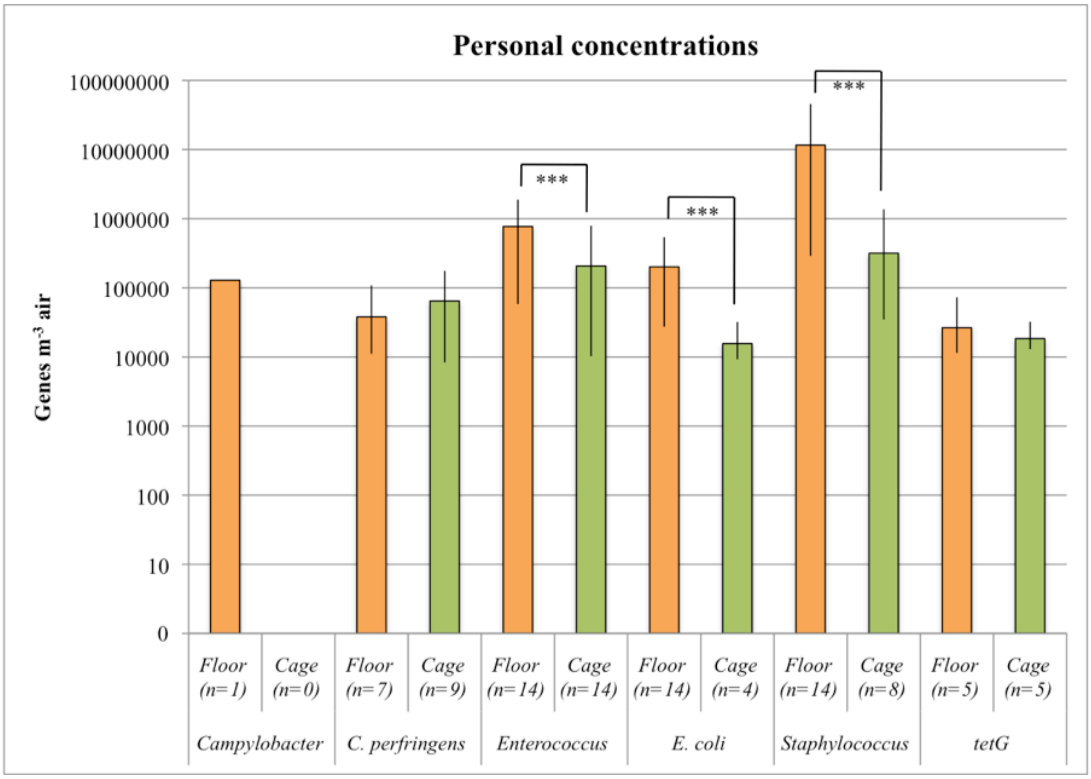

Figure 1B. Personal concentrations. Bacteria and *tet*G concentrations were determined in bioaerosols from floor-housed (FH) and cage-housed (CH) poultry operations collected with area and personal samplers. Data is reported as genes per m^3 air (*16S rRNA* for *C. perfringens*, *Campylobacter*, *E. coli* and *Enterococcus*, *tuf* for *Staphylococcus* and *tet*G for tetracycline resistance). The number of positive samples is indicated (*n*). Target genes, primers, hybridization temperatures and amplicon lengths for quantitative PCR are indicated in Table I. Raw data, averages with ranges indicated, is presented in the graphs. Data was log transformed prior to statistical analyses. ***$p<0.001$

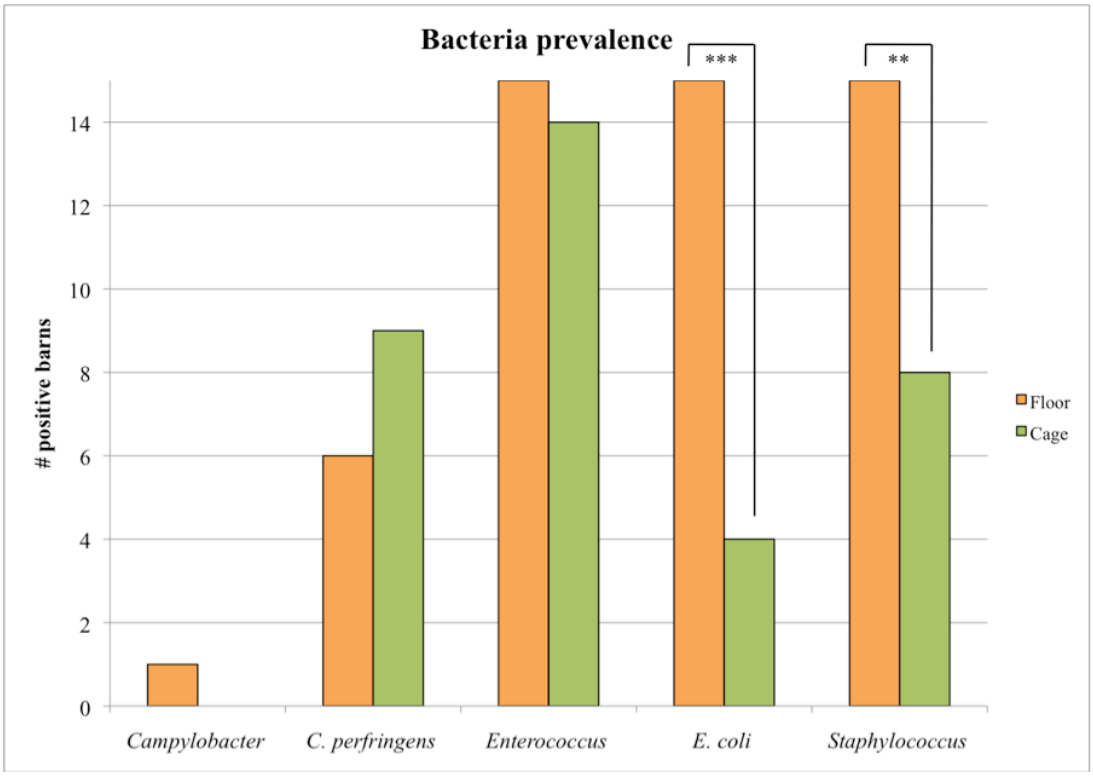

Figure 2. Bacteria prevalence. Bacteria were detected in bioaerosols from floor-housed (FH) and cage-housed (CH) poultry operations collected with area and personal samplers using quantitative PCR. Target genes, primers, hybridization temperatures and amplicon lengths are indicated in Table I. The number of FH and CH barns positive for each organism is indicated. Barns were considered positive if bacteria were detected in both area and personal samples. **p<0.01 **p<0.001

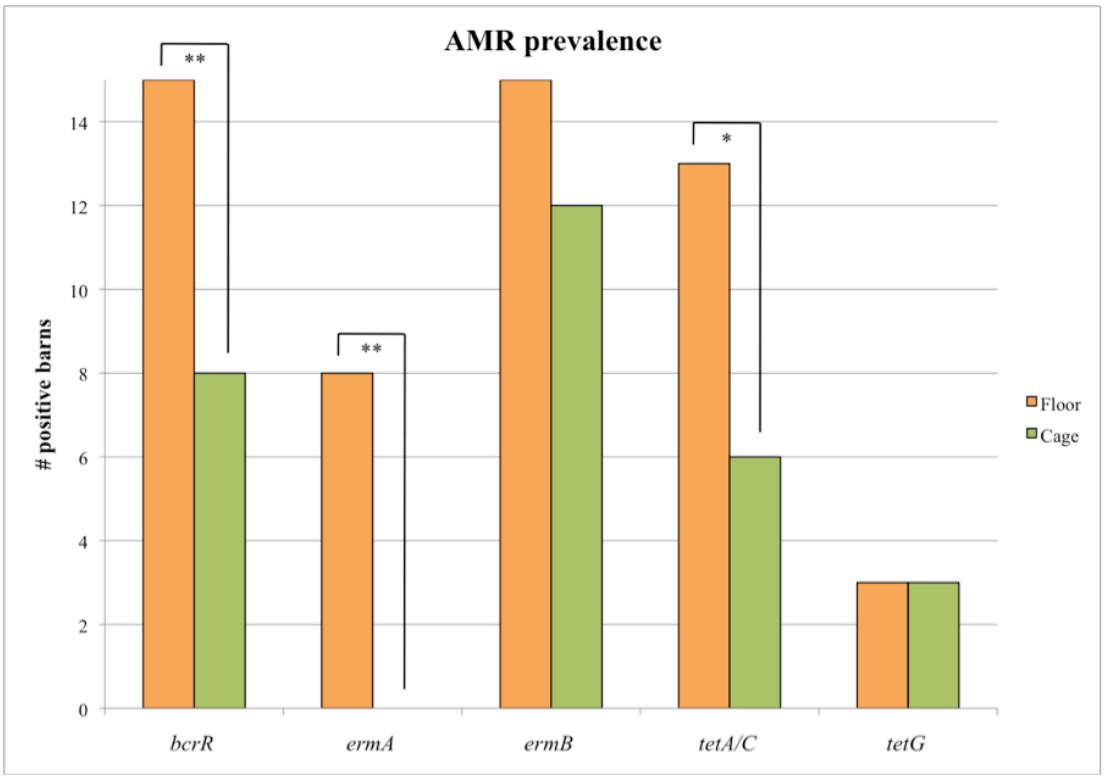

Figure 3. Antimicrobial resistance (AMR) prevalence. AMR genes were detected in bioaerosols from floor-housed (FH) and cage-housed (CH) poultry operations collected with area and personal samplers using end-point PCR. Target genes, primers and hybridization temperatures are indicated in Table I. The number of FH and CH barns positive for each AMR gene is indicated. Barns were considered positive if AMR genes were detected in both area and personal samples. *$p<0.05$ **$p<0.01$

4.5 Discussion

Staphylococcus was the most concentrated of detected organisms and was significantly more concentrated in FH poultry operations. The detection of high *Staphylococcus* concentrations in bioaerosols from FH operations, where litter is used, is supported by a previous study that found a predominance of *Staphylococcus* in the presence of litter [4]. The next most highly concentrated organisms detected were *Enterococcus* and *E. coli*, which were significantly higher in FH bioaerosols. Previous studies using culture-dependent techniques also detected *Enterococcus* [104] and *E. coli* [105] in poultry bioaerosols. *Campylobacter* was detected only in bioaerosols from FH facilities. Low concentrations of *Campylobacter* are supported by previous studies that either did not detect *Campylobacter* [104] or detected *Campylobacter* only once [105] in poultry bioaerosols. *C. perfringens* was the only bacteria of those examined found to be more concentrated in CH bioaerosols and this difference was only significant in area bioaerosols. *Salmonella* was not detected in poultry bioaerosols in this study (data not shown). This observation is supported by studies that rarely detected *Salmonella* [104, 105] or did not detect *Salmonella* in bioaerosols from poultry houses without prior salmonellosis [106].

Previously published biodiversity studies detected *Staphylococcus cohnii* in CH and FH bioaerosols [95]. *Staphylococcus* isolates from poultry operations, including *S. cohnii*, have been shown to harbour erythromycin-resistant methylase (erm) genes, *erm*A and *erm*C [117, 118]. Although *Staphylococcus* was detected in both FH and CH bioaerosols, the *erm*A resistance gene was detected only in FH bioaerosols. *Campylobacter* has also been shown to harbour erythromycin resistance [36-38] and was

detected only in FH bioaerosols. *Campylobacter*, *E. coli* and *Staphylococcus* isolates from poultry operations have been shown to harbour resistance genes against tetracycline [32, 36-38, 117]. Although there was no significant difference in tetracycline resistance gene, *tet*G, concentrations between FH and CH bioaerosols, tetracycline resistance gene, *tet*A/C, was detected at a higher prevalence in FH bioaerosols. *Enterococcus* is known to display multi-AMR [36-38]. *Enterococcus* isolates from poultry operations have been shown to harbour zinc bacitracin resistance gene, *bcr*R, and erythromycin resistance gene, *erm*B, which can also confer resistance to group B compounds such as virginiamycin [36]. Although *Enterococcus* and *erm*B were highly prevalent in both FH and CH bioaerosols, all FH operations were positive. Zinc bacitracin resistance gene, *bcr*R, was also detected in every FH facility and at a higher prevalence than in CH operations. Every barn that was positive for *bcr*R was also positive for *erm*B. These observations suggest that use of growth promoting antibiotics, which is limited to FH operations, may contribute to higher prevalences of erythromycin resistance, tetracycline resistance and zinc bacitracin resistance. It is possible that resistance to erythromycin and zinc bacitracin may be acquired through multi-AMR genes. However, there is not enough information from the current study to make a direct link between specific antibiotic use and the presence of antimicrobial resistance at individual facilities.

Another difference between CH and FH poultry operations is manure management, which may play a role in the observed variation of bacteria prevalence and AMR prevalence in bioaerosols. It is possible that fecal bacteria and harboured resistance genes are more easily aerosolized in FH poultry facilities, where litter is used, than CH operations, where manure is stored. Therefore, the use of litter, which is limited

to FH facilities, may contribute to higher prevalences of erythromycin resistance, tetracycline resistance and zinc bacitracin resistance in bioaerosols.

In addition to fecal bacteria harbouring AMR genes being a source of dust in food production facilities, the antibiotic-treated feed particles may also be a source of dust. Antibiotics have been isolated from inhalable and respirable dust sizes in swine production facilities [48, 49]. It has been shown that the number of hours spent in a swine production facility is associated with AMR [41]. The presence of antimicrobial genes in poultry bioaerosols suggests that poultry farmers may be potential nasal carriers of antimicrobial resistant bacteria, which could potentially be transmitted to susceptible persons. It has already been suggested that swine workers are nasal carriers of resistant bacteria, where pathogenic bacteria and tetracycline resistance genes were detected in bioaerosols from swine confinement buildings and nasal swabs from swine workers [110]. Similar observations have been made in health care workers, who are known to be nasal carriers of methicillin-resistant *Staphylococcus aureus* (MRSA) that can be transmitted to immunocompromised patients [119]. Treatment with intranasal antibiotics to eliminate bacteria in nasal carriers has been shown to help reduce respiratory transmission [120]. It is becoming more evident that workers, producers and farm residents are potential reservoirs of multiple AMR genes [41].

There are few studies in the literature that have examined the presence of specific bacteria, including *Jeotgalicoccus* spp. [58], *Staphylococcus* spp. [4, 104], *Enterococcus* spp. [104], *Campylobacter* spp. [104, 105], *Salmonella* spp. [104-106], *C. perfringens* [104] and *E. coli* [105], in poultry bioaerosols. Although molecular techniques are commonly used to characterize AMR genes, most research relies on culture-dependent

characterization of bacteria that may underestimate numbers of bacteria [4, 57, 106], especially from aerosol samples where cell survival may be compromised [105]. The current study utilizes culture-independent methods to quantify bacteria, as well as identify AMR genes, in poultry house bioaerosols. Finally, few studies exist that compare the environments between CH and FH poultry facilities. It has been shown that management practices, worker symptoms and total dust vary significantly between these two housing types [1, 77, 95], suggesting that occupational exposures may differ. To the authors' knowledge, this is the first direct comparison of pathogens and AMR genes in bioaerosols from CH and FH poultry operations.

Resistance to antibiotics is becoming a public health concern. It is predicted that our supply of new, safe, effective and affordable antimicrobials is finite. It has been shown that use of antibiotics in food production is a contributing factor to AMR [33]. Although there are obvious benefits to the use of therapeutic antibiotics in food production, Health Canada is undergoing assessment of the benefits of sub-therapeutic antibiotic use [32]. Hence, a more sustainable approach to preventing AMR health issues is necessary, including AMR surveillance programs and exposure assessments. This assessment of AMR in poultry bioaerosols provides insight to the possible respiratory exposure of poultry workers to AMR bacteria and is an important contribution to the existing knowledge base.

4.6 Conclusions

Campylobacter, *Enterococcus*, *E. coli* and *Staphylococcus* were more concentrated and *E. coli* and *Staphylococcus* were more prevalent in bioaerosols from FH

operations than CH bioaerosols. These bacteria are potentially pathogenic to humans and have been shown to harbour AMR against tetracycline, erythromycin, virginiamycin and zinc bacitracin. Zinc bacitracin resistance gene, *bcr*R, erythromycin resistance gene, *erm*A, and tetracycline resistance gene, *tet*A/C, were more prevalent in FH bioaerosols than bioaerosols from CH facilities. Virginiamycin and zinc bacitracin are commonly used as growth promoters in FH operations. These data suggest that the use of growth-promoting antibiotics in FH poultry facilities may contribute to the presence of AMR bacteria in bioaerosols.

4.7 Acknowledgements

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5.0 DISCUSSION

5.1 General discussion

Modern methods of poultry facility management require that workers spend a large proportion of the day in an atmosphere containing comparatively high levels of dust, gases and odours [7]. Studies of nine different industries showed the highest prevalence of work-related lower and upper respiratory symptoms and chronic bronchitis in poultry workers [78]. Inhalation of poultry bioaerosols can result in allergic, toxic or inflammatory responses (asthma-like syndrome or bronchitis) in workers [59, 121]. Poultry workers suffer from current and chronic respiratory symptoms, including cough, wheeze and phlegm, and cage-housed (CH) workers have reported a higher prevalence of some of these symptoms [1, 63]. Indoor air from poultry facilities ventilates to the outdoors, compromising ambient air quality [121]. There is an increased incidence of respiratory illness in neighboring communities and bacteria are present at higher concentrations downwind from food production facilities [45]. These bacteria are also shown to be higher in antimicrobial resistance than bacteria found upwind of food production operations [34, 46, 47]. Therefore, understanding the components of poultry barn air is not just a concern for the poultry workers, but also of public health.

Poultry barn dust consists of: 1. gases – carbon dioxide, ammonia and hydrogen sulfide, 2. inorganic particulates – soil and dust, 3. nonviable organic particulates – feces,

urine, feathers, dander, litter, and feed, including antibiotic-treated feed particles and 4. viable organic particulates – grain mites, spores, pollens, bacteria, fungi, viruses, archaea, and their byproducts – fragments, endotoxin, β-glucan, peptidoglycan and DNA, including antimicrobial resistance genes (ARGs) [1, 6, 7, 48, 56, 121]. The purpose of this research project was to investigate bacteria, archaea and ARGs in poultry bioaerosols and compare these components in two types of poultry operations, cage-housed (CH) and floor-housed (FH) facilities. Total bacteria, *Enterococcus* spp., *E. coli* and *Staphylococcus* spp. were significantly higher and *Campylobacter* spp., zinc bacitracin resistance gene, *bcr*R, erythromycin resistance gene, *erm*A, and tetracycline resistance gene, *tet*A/C, were more prevalent in bioaerosols from FH poultry operations than CH bioaerosols. *C. perfringens* and methanogenic archaea were significantly higher in bioaerosols from CH poultry operations than FH bioaerosols.

Previous studies suggest that a significant portion of bioaerosols originate from feces [57, 121]. Therefore, any characteristic that may affect fecal microbiota such as age and type of animal, which differ between CH and FH poultry houses, could influence the biodiversity of bioaerosols. However, a recent study investigated bioaerosols released from chicken feces samples in a controlled environment and found less *Staphylococcus* spp. than studies where bioaerosols were collected from poultry barns [56]. Therefore, *Staphylococcus* spp. found in poultry barn air may originate from a source other than feces, such as feed, feathers or dander. *Staphylococcus* spp. increases during the growth cycle of birds [4], suggesting that feathers and/or dander that shed during the growth cycle may be a source of *Staphylococcus* spp. in FH operations, which house younger birds than CH facilities.

In contrast to total bacteria concentrations, which were significantly higher in bioaerosols from FH barns, archaea were more concentrated in bioaerosols from CH poultry operations and in many FH bioaerosol samples archaea were undetected. Similarly, higher concentrations of archaea were detected in ceca samples from layer hens than in fecal samples from broiler birds [26, 27]. Archaea found in the gut metabolize fermentation products, such as alcohols, organic acids, CO_2 and H_2, and produce methane [25]. Many of these substrates are first processed by bacteria prior to methanogenesis [24]. Methanogenic archaea enhance the growth and activity of bacterial polysaccharide consumers, such as bacteroides and firmicutes [22]. An increase of polysaccharide consumers may indirectly promote caloric intake and fat accumulation [22], acting as a "natural" growth promoter. The presence of archaea can have an effect on other fecal microbiota and the differential archaea concentrations between CH and FH operations may influence the biodiversity of bioaerosols.

The use of antibiotics, which differs between CH and FH facilities, has been shown to affect fecal microbiota [81], influencing bioaerosol biodiversity. In our study, six of the 15 FH operations reported the use of antibiotics in feed or water while none of the CH facilities reported antibiotic use. Of the six FH operations using antibiotics, three DGGE profiles (28, 29 and 30) clustered together and two DGGE profiles (21 and 22) clustered together. Previous PCR-DGGE studies revealed that antibiotics alter chicken intestinal microbiota [81, 82] and DGGE profiles cluster based on antibiotic treatment [83], supporting our results.

Not only does the use of antibiotics alter biodiversity, it can also lead to antimicrobial resistant microorganisms [108]. Antimicrobial resistance, which adversely

affects human health, can be acquired through long-term inhalation of antibiotics [41], direct transfer of antimicrobial resistance genes (ARGs) from zoonotic infections or the indirect transfer of ARGs from commensal animal bacteria to human pathogens [40]. Our investigation of bacterial diversity in poultry bioaerosols identified bacterial species that are known to harbour antimicrobial resistance (*Campylobacter* spp., *Enterococcus* spp., *E. coli* and *Staphylococcus* spp.) or disinfectant resistance (*Sphingomonas* spp.) There is concern that disinfectant resistance could contribute to antimicrobial resistance through co-selection of resistance genes [93]. Just as bacteria acquire AMR, archaea can become resistant to antimicrobials. Archaea are susceptible to antimicrobials that are effective against bacteria and eukaryotes such as bacitracin, an antibiotic that targets the lipid cycle and is commonly used in the poultry industry [22, 100]. Transmission of microorganisms and ARGs from animals to humans can occur through direct contact, contaminated water, environment and food [41]. Detection of bacterial DNA, archaeal DNA and ARGs in poultry bioaerosols indicates that workers may be inhaling microorganisms or free DNA containing ARGs. Therefore, another route of transmission—respiratory—may be possible.

Although our findings cannot be directly related to poultry worker health, they provide a greater understanding of the poultry barn air environment. The following threshold values for poultry facilities have been proposed: 2.5 mg/m^3 total dust and 600 EU/m^3 total endotoxin [8]. We detected average area dust concentrations for FH and CH bioaerosols of 4.5 and 1.8 mg m^{-3}, respectively [77], and average personal dust concentrations of 5.1 and 1.9 mg m^{-3}, respectively. The average total dust concentrations from FH operations exceed the proposed threshold values. We detected average area

endotoxin concentrations for FH and CH bioaerosols of 2.9 x 10^3 and 1.9 x 10^3 EU m^{-3}, respectively and average personal endotoxin concentrations of 3.4 x 10^3 and 2.0 x 10^3 EU m^{-3}, respectively. Average endotoxin concentrations well exceed the proposed threshold values. To date, there are no culture-independent proposed threshold limit values for exposure to bacteria, archaea or ARGs in poultry houses.

Although total dust, endotoxin and bacteria levels are high in poultry bioaerosols, they do not help to explain the greater prevalence of current and chronic phlegm in CH workers. However, biodiversity studies reveal that bacterial profiles are significantly different between personal bioaerosols from CH and FH operations. Sequences affiliated with *Mycobacterium* sp. were more prevalent in CH personal biodiversity profiles. Mycobacterial bioaerosols from metalworking fluids, swimming pools, hot tubs and water-damaged buildings are reported to cause respiratory diseases, including hypersensitivity pneumonitis. It is possible that *Mycobacterium* sp. may play a role in the greater prevalence of respiratory symptoms in CH workers. Of the specific bacterial species investigated, only *C. perfringens* were more concentrated in CH bioaerosols. Although it is commonly known to cause foodborne illness, *C. perfringens* can result in pulmonary infection [122]. Thus, the greater concentrations of *C. perfringens* in CH bioaerosols may help to explain the greater prevalence of current and chronic phlegm in CH workers.

Archaea were detected in most CH bioaerosol samples but in only half of the FH bioaerosols. Overall, archaea concentrations were significantly higher in bioaerosols from CH facilities. Methanogenic archaea have been detected in bioaerosols from swine [29] and dairy barns [28]. Two methanogens, *Methanobrevibacter smithii* and

Methanosphaera stadtmanae, have been shown to cause immunogenic responses in mice following intranasal exposure [30]. Therefore, the greater prevalence and concentrations of archaea in CH bioaerosols may play a role in the greater prevalence of respiratory symptoms in CH workers.

Data from chapter two "Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations" revealed that personal levels of dust, endotoxin, and bacteria were significantly higher in FH bioaerosols than CH bioaerosols despite previous results that detected a trend of higher endotoxin concentrations in CH bioaerosols [1]. Biodiversity profiles from CH and FH personal bioaerosols shared less than 20% similarity. The results from chapter two suggest that total personal dust, endotoxin or bacteria concentrations may not explain the higher prevalence of respiratory symptoms in CH workers, so other bioaerosol components were examined. Data from chapter three "Archaeal characterization of bioaerosols from cage-housed and floor-housed poultry operations" showed that CH workers are exposed to higher levels of airborne archaea than FH workers and this may contribute to the greater prevalence of respiratory symptoms in CH workers. Results from chapter two also suggest that bacterial diversity may help to explain the higher prevalence of respiratory symptoms in CH workers, that antibiotic use may influence biodiversity, and that bacteria known to harbour antimicrobial resistance genes (ARGs) are detected in bioaerosols from CH and FH poultry barns. Studies from chapter four "Potentially pathogenic bacteria and antimicrobial resistance in bioaerosols from cage-housed and floor-housed poultry operations" detected a greater prevalence of zinc bacitracin resistance gene (*bcr*R), erythromycin resistance gene (*erm*A), and tetracycline resistance gene (*tet*A/C) in

bioaerosols from FH facilities. Of the bacteria examined, all except *C. perfringens* were more concentrated in bioaerosols from FH operations, where growth-promoting antibiotics are used. *C. perfringens* may play a role in the greater prevalence of respiratory symptoms in CH workers.

5.2 Limitations of the study

A common molecular method to detect bacteria and archaea is PCR amplification of the 16S rRNA gene followed by denaturating gradient gel electrophoresis and sequencing. The 16S rRNA gene is ideal because of its universality, slow rate of evolution, and alternating conserved and variable regions. However, the slow rate of evolution means that closely related organisms have nearly identical sequences and the 16S rRNA gene has a regular occurrence of insertions and deletions [103]. PCR using primers targeting different variable regions of the bacterial 16S rRNA gene can result in different DGGE profiles and different primer sets also result in different sequences. One study showed that amplification of the variable V3 region (344-519) resulted in the most well separated bands and recovered common sequences [102]. Protein-encoding genes may be a better option because they are present as single copies in prokaryotic genomes, they have low rates of insertions and deletions, and they accumulate silent mutations (due to codon degeneracy) resulting in better species resolution [103]. Detection of *rpo*B, the β subunit of RNA polymerase, has greater specificity than 16S rRNA due to greater sequence divergence between species [25]. For detecting bacteria, type I chaperonin (*cpn*60) is the most developed alternative to 16S rRNA [103]. There are 'universal' primers, a large sequence database and it has great discriminating power. For archaea

detection, type II chaperonin (thermosome, homologue to *cpn*60) is suggested [103]. When PCR targeting thermosome was compared to 16S rRNA and *mcr*A, all detected the same major species but greater distinction was achieved with thermosome-targeted PCR [103]. To date, there are fewer thermosome sequences in the database.

Another limitation of molecular techniques that detect bacteria, archaea and/or ARGs in bioaerosols is that we cannot answer the following questions:

Is the organism alive or dead?

Does long-term inhalation of the organism lead to respiratory symptoms?

Is the organism harbouring the ARG alive or dead?

Is the ARG expressed?

Is free DNA transforming into viable organisms?

Does long-term inhalation of ARGs lead to AMR in respiratory tract flora? [121]. Although culture-based techniques may underestimate bacteria concentrations in bioaerosols, detection of ARGs from cultured bacteria is necessary to establish a link between bacteria species and the ARGs that they harbour.

Studies that examine bioaerosols collected on-site such as ours can be used to assess worker exposure but cannot confirm the source of bioaerosols.

5.3 Recommendations from the study

Our findings indicate that poultry barns do not provide a safe indoor air environment for workers and possible modes of remediation include: removing sources (if possible), reducing aerosolization, reducing exposures, and treating responses. Use of pelleted food, routine entry into buildings and lighting cycles can reduce aerosolization of

dust [7]. Spraying canola oil to reduce dust exposure has shown to be beneficial in reducing acute health effects of naïve subjects following a five-hour exposure in a swine facility [11]. Endotoxin antagonists may play a therapeutic role in preventing or reducing endotoxin-induced environmental airway disease [74]. Use of well-fitted N-95 respirators over a four-hour exposure to swine barn air reduced the inflammatory response, as measured by elimination of acute respiratory symptoms, shift changes, lung function response to methacholine inhalation, serum IL-6 response and nasal lavage IL-6 and IL-8 responses [73]. It is recommended that poultry workers use National Institute Occupational Safety and Health-approved particle respirators to reduce their exposure to poultry bioaerosols containing dust, endotoxin, bacteria, archaea, and ARGs [46].

Future discoveries in this area may lead to changes in Canadian poultry production in order to reduce the transmission of AMR. The benefits of growth promoters are generally greater in poor hygienic conditions, suggesting that management practices (probiotics, vaccines, biosecurity) could reduce the need for antimicrobials [34]. It is also suggested that new breeds of animals could reduce the need for antibiotic growth promoters [34]. If antibiotic use is required, rotation of the types of antibiotics used within a facility is recommended [46]. Finally, the recommendation to phase out the use of antibiotics as growth promoters is gaining popularity [34, 46].

6.0 CONCLUSIONS

Investigations of poultry house environments do not often distinguish between the two types of operations, cage-housed (CH) and floor-housed (FH). A few studies have compared total dust between the two barn types and respiratory symptoms between the two types of workers. Although total dust concentrations are higher in FH facilities, CH workers report a greater prevalence of symptoms. The purpose of this project was to examine several bioaerosol components (bacteria, archaea, ARGs) and to compare bioaerosols from CH and FH poultry houses in order to better understand the nature of poultry worker exposures in these environments. We detected higher concentrations of total bacteria, *Enterococcus* spp., *E. coli*, *Staphylococcus* spp., and *Campylobacter* spp. in bioaerosols from FH poultry operations than CH bioaerosols and higher concentrations of methanogenic archaea and *C. perfringens* in bioaerosols from CH facilities. Biodiversity studies revealed that personal bioaerosol profiles from FH operations share less than 20% similarity with profiles from CH barns. Reported antibiotic use is higher in FH facilities and we detected higher prevalences of antibiotic resistance genes including zinc bacitracin resistance gene, *bcr*R, erythromycin resistance gene, *erm*A, and tetracycline resistance gene, *tet*A/C.

The way that birds are housed results in key differences in the aerobiology between these two barn environments. There are different **sources** of bioaerosols. CH

facilities house predominantly female birds that are older than FH birds and lay eggs. Young birds of both sexes in FH operations undergo a moulting phase. When birds are housed on the floor the use of litter is required. **Aerosolization** factors are different. Bird activity and movement is much more limited when birds are housed in cages. When birds are housed on the floor their movement aerosolizes litter and fecal matter. This study, along with previous research, has highlighted differences in **exposures** to dust, endotoxin, bacteria, archaea, and ARGs between these two barn environments. FH workers are exposed to higher levels of dust, endotoxin, bacteria, and ARGs while CH workers are exposed to higher levels of archaea. Previous studies have shown that **responses** to occupational exposures differ between CH and FH workers. CH workers report a greater prevalence of respiratory symptoms. Specific **remediation** strategies for each barn type may be required.

7.0 FUTURE DIRECTIONS

Our study detected significant differences in bacterial diversity between CH and FH poultry bioaerosols. It was shown that inflammatory cells treated with Gram-negative or Gram-positive bacteria produce different inflammatory cytokine profiles [19]. The differences in reported respiratory symptoms between workers from CH and FH operations may be explained by the production of different inflammatory cytokines in the lung. It will be necessary to further examine the quantities of Gram-positive and Gram-negative bacteria in bioaerosols from CH and FH poultry operations. A comparison of inflammatory cytokine profiles in cells treated with CH or FH bioaerosols is also required.

The higher prevalence of reported respiratory symptoms in CH vs. FH workers may also be explained by the higher concentrations of archaea in CH bioaerosols. It will be necessary to examine the response of airborne archaea detected in poultry bioaerosols, especially *Methanobrevibacter woesei*, on respiratory health.

In order to determine if poultry workers are carriers of ARGs, it will be necessary to examine the nasal flora of workers. A comparison of nasal flora from FH vs. CH workers will aid in understanding the risks of AMR transmission associated with growth promoters. Also, it will be important to investigate the presence of ARGs in exhaust air from poultry facilities to assess the risk of AMR transmission outside the facilities.

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APPENDIX A: Cellular responses to poultry dust extract from bioaerosols

A.1 Introduction and methods

Previous studies have shown that treatments with 5% hog dust extract (HDE) stimulate a release of IL-8 from bronchial epithelial cells greater than the release following exposure to LPS alone [67]. In these studies, HDE was prepared by diluting 1g of settled dust from a hog barn in 10mL Hanks' balanced salt solution. In our study, dust fractions (usually less than 1mg) were collected on PVC filters using a Marple cascade impactor. The filters containing dust were added to 10mL endotoxin-free water, supplied with the LAL assay kit.

A comparison of cellular responses following exposures to bioaerosols from CH and FH poultry operations would greatly add to this work. Attempts were made to do so but, unfortunately, were unsuccessful.

A.2 Results and discussion

Calu-3 bronchial epithelial cells produced approximately 18-fold more IL-8 mRNA following 2h LPS exposure (1μg/mL) than untreated cells (Figure 1). Although the endotoxin concentrations in our 50% poultry dust extracts are low (0.2μg/mL), Calu-3 cells produced approximately 5-fold more IL-8 mRNA following LPS exposure at this concentration than untreated cells (Figure 1). Calu-3 cells did not produce IL-8 mRNA following 5% and 10% poultry dust extract exposure (data not shown) and produced

approximately 3-fold more IL-8 mRNA following 50% poultry dust extract exposure (Figure 1). However, an approximate 2-fold increase in IL-8 mRNA production was observed from Calu-3 cells treated with 50% endotoxin-free water alone (Figure 1).

A.2.1 Is water a problem?

Calu-3 cells respond similarly whether they are treated with 50% PDE prepared in endotoxin-free water, 50% endotoxin-free water alone, 50% clean filter extract prepared in endotoxin-free water, co-treated with 50% endotoxin-free water plus 0.2μg/mL LPS, or co-treated with 50% clean filter extract prepared in endotoxin-free water plus 0.2μg/mL LPS (Figure 1). In order to investigate whether treating cells with 50% endotoxin-free water was altering their ability to respond to LPS, Calu-3 cells were treated with 5μg/mL LPS alone, 50% endotoxin-free water alone, 50% endotoxin-free water plus 5μg/mL LPS, or 50% clean filter extract plus 5μg/mL LPS. Calu-3 cells treated with 5μg/mL LPS produced approximately 18-fold more IL-8 mRNA than untreated cells (Figure 2). Calu-3 cells treated with 50% endotoxin-free water produced approximately 2-fold more IL-8 mRNA than untreated cells (Figure 2). Cells treated with 50% endotoxin-free water plus 5μg/mL LPS produced approximately 6-fold more IL-8 mRNA than untreated cells and cells treated with 50% clean filter extract plus 5μg/mL LPS produced approximately 4-fold more IL-8 mRNA than untreated cells (Figure 2). The normal production of IL-8 mRNA from Calu-3 cells in response to LPS exposure is inhibited by co-treatment with 50% endotoxin-free water or 50% clean filter extract prepared in endotoxin-free water.

A.2.2 Is the amount of water or lack of salinity a problem?

In order to examine whether the lack of salinity in water prevents cells from responding normally to LPS, Calu-3 cells were treated with 50% endotoxin-free water or PBS, 50% endotoxin-free water or PBS plus 5μg/mL LPS, or 50% clean filter extract prepared in endotoxin-free water or PBS plus 5μg/mL LPS. Calu-3 cells treated with 50% endotoxin-free water or 50% PBS produced approximately 2-fold more IL-8 mRNA than untreated cells (Figure 2). Calu-3 cells treated with 50% endotoxin-free water plus 5μg/mL LPS produced approximately 6-fold more IL-8 than untreated cells, whereas cells treated with 50% PBS plus 5μg/mL LPS produced approximately 8-fold more IL-8 than untreated cells (Figure 2). Also, Calu-3 cells treated with 50% clean filter extract prepared in endotoxin-free water plus 5μg/mL LPS produced approximately 4-fold more IL-8 mRNA than untreated cells, while cells treated with 50% clean filter extract prepared in PBS plus 5μg/mL LPS produced approximately 10-fold more IL-8 mRNA than untreated cells (Figure 2). Therefore, treating cells with 50% water or salt solution interferes with the normal response to LPS. Dust extracts need to be more concentrated so that cells can be treated at 5% dust extract, as was done in other studies [67]. Since co-treatment with PBS caused less interference with the normal response to LPS than co-treatment with endotoxin-free water, it is recommended that dust extracts be prepared with a salt solution rather than water.

A.2.3 Is dust or endotoxin binding to the filter?

It was hypothesized that LPS may bind to the PVC filter and, despite vortexing, not be released into the dust extract. However, when Calu-3 cells were co-treated with 50% clean filter extract (prepared with endotoxin-free water or PBS) and LPS (5μg/mL), the normal production of IL-8 mRNA (approximately 18-fold) was reduced to approximately 4-fold and 10-fold above untreated cells, respectively (Figure 2). Therefore, the interference with normal cellular responses to LPS is not likely caused by LPS binding to the PVC filter. It is possible that something in the PVC filter is dissociated and dissolved in the water during the vortex step of dust extract preparation, and that this "something" may interfere with the normal cellular response to LPS or dust.

A.2.4 Is the expression of other cytokines induced?

Calu-3 bronchial epithelial cells produced approximately 50-fold more TNF-α mRNA following 2h LPS exposure (1μg/mL) than untreated cells (Figure 3). Calu-3 cells did not produced TNF-α mRNA following 10% poultry dust extract exposure (data not shown) and produced approximately 3-fold more TNF-α mRNA following 50% poultry dust extract exposure (Figure 3). However, an approximate 3-fold increase in TNF-α mRNA production was also observed from Calu-3 cells treated with 50% clean filter extract prepared in endotoxin-free water (Figure 3). Calu-3 cells treated with 50% clean filter extract plus 5μg/mL LPS produced approximately 19-fold more TNF-α mRNA than untreated cells (Figure 3). Similarly to the production of IL-8, the normal production of TNF-α mRNA from Calu-3 cells in response to LPS exposure is inhibited by co-treatment with 50% clean filter extract prepared in endotoxin-free water.

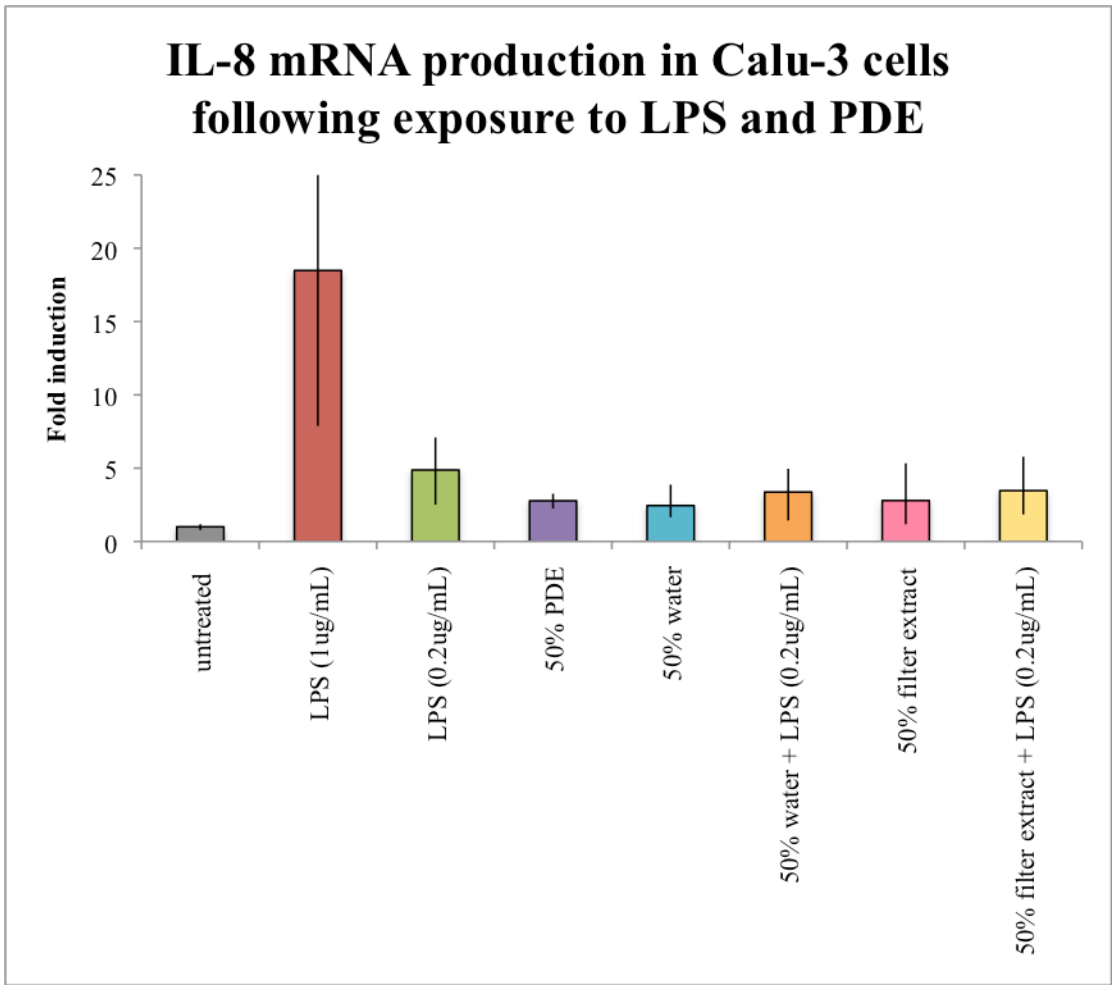

Figure 1. IL-8 mRNA production in Calu-3 cells treated for 2h with 1μg/mL LPS, 0.2μg/mL LPS, 50% poultry dust extract in endotoxin-free water, 50% endotoxin-free water, 50% endotoxin-free water plus 0.2μg/mL LPS, 50% clean filter extract in endotoxin-free water, or 50% clean filter extract in endotoxin-free water plus 0.2μg/mL LPS. IL-8 mRNA production was measured using qRT-PCR.

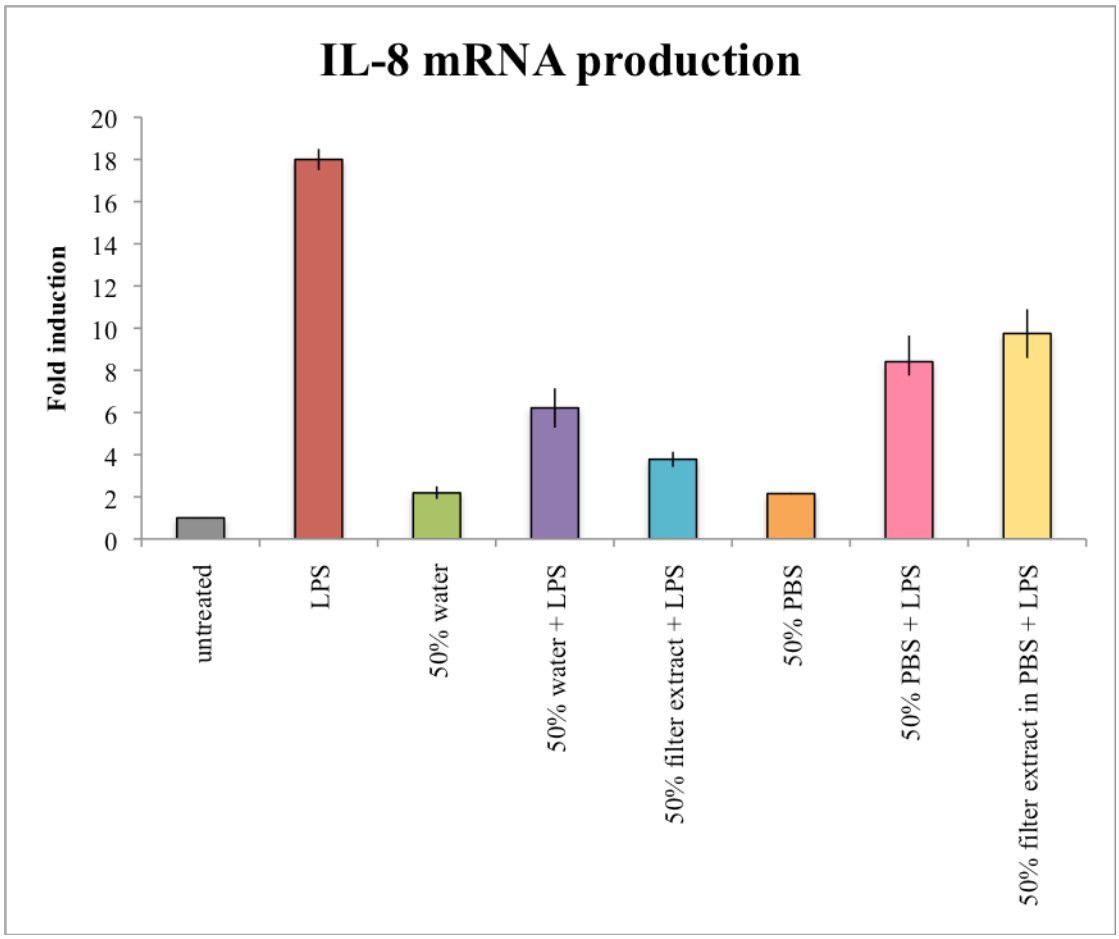

Figure 2. IL-8 mRNA production in Calu-3 cells treated for 2h with 5µg/mL LPS, 50% endotoxin-free water plus 5µg/mL LPS, 50% clean filter extract in endotoxin-free water plus 5µg/mL LPS, 50% PBS plus 5µg/mL LPS, or 50% clean filter extract in PBS plus 5µg/mL LPS. IL-8 mRNA production was measured using qRT-PCR.

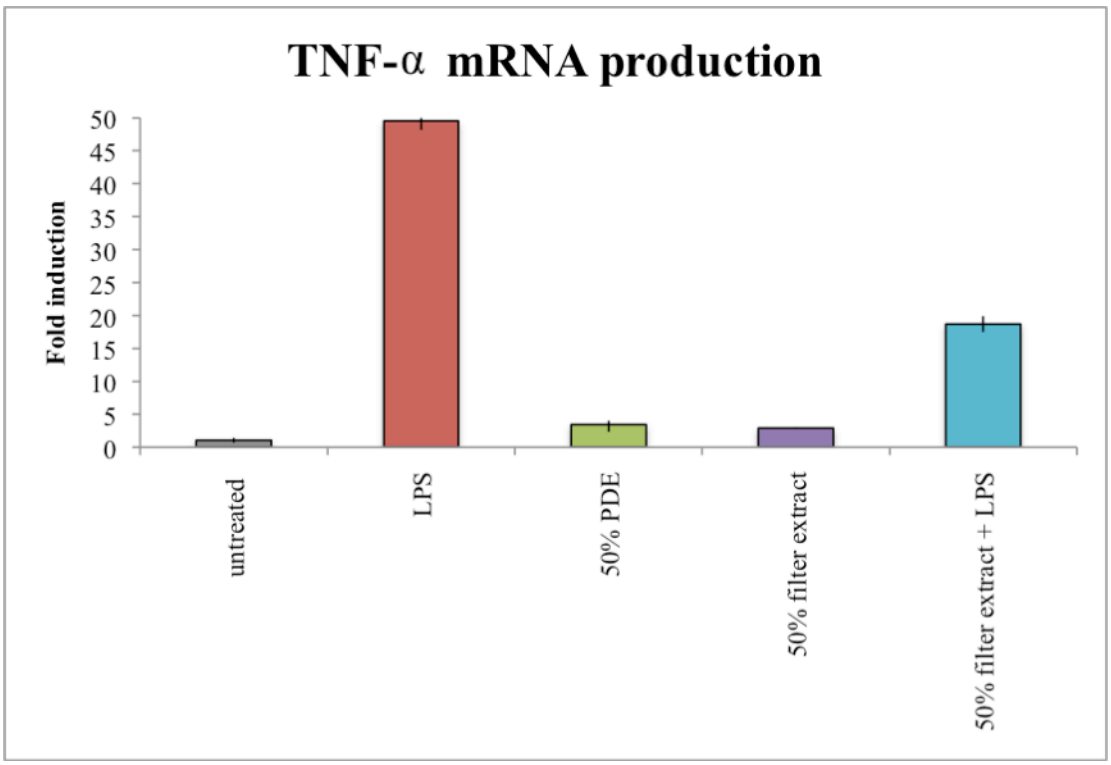

Figure 3. TNF-α mRNA production in Calu-3 cells treated for 2h with 1μg/mL LPS, 50% poultry dust extract in endotoxin-free water, 50% clean filter extract in endotoxin-free water, or 50% clean filter extract in endotoxin-free water plus 5μg/mL LPS. TNF-α mRNA production was measured using qRT-PCR.

A.3 Conclusions

Our preliminary results suggest that our samples were not collected in an appropriate fashion for cellular-based experiments. Diluting fractioned dust in 10mL endotoxin-free water resulted in low dust extract concentrations. Using water rather than a salt solution to prepare dust extracts may interfere with cellular responses. Adding PVC filters and dust to extract liquid may also interfere with cellular responses.

APPENDIX B: Cellular responses to poultry dust extract from settled dust

Since attempts to examine cellular responses to poultry dust extracts prepared from bioaerosol samples were unsuccessful, cellular responses to poultry dust extract prepared with settled dust were examined.

B.1 Introduction

Studies of nine different industries showed that poultry workers suffer the highest prevalence of work-related lower and upper respiratory symptoms and chronic bronchitis [1]. The respiratory symptoms observed in workers characterize an asthma-like syndrome, bronchitis, or exacerbation of pre-existing asthma [59]. Workers typically complain of chronic cough that may be accompanied by sputum, chest tightness, shortness of breath with exertion and wheezing [1].

Poultry farmers have a higher exposure to environmental factors such as dust, endotoxin and microorganisms [7, 50]. Dust is a complex mixture of particles of organic and inorganic origin and different gases absorbed in aerosol droplets. The sources of dust from a poultry facility include: dried fecal matter and urine, skin flakes, feathers, grain mites, spores, pollens, feed and litter particles, fungal constituents, viruses, bacteria, ammonia and endotoxin [1, 5-7]. The solid components of dust act as a transport vector for noxious gases (such as ammonia), microbial products and components (such as endotoxin, β-glucan and peptidoglycan), allowing these to be inhaled into the lungs [8].

Lung epithelial cells are some of the first cells exposed to inhaled bioaerosols. Upon interaction with inhaled irritants, epithelial cells release inflammatory mediators, including cytokines TNF-α, IL-6 and IL-8. IL-8 is important for the recruitment of neutrophils to the site of inflammation [67]. Endotoxin alone can induce this inflammatory pathway. However, a previous study showed that epithelial cells released more IL-6 and IL-8 cytokines following exposure to HDE than LPS alone [67]. Therefore, it was hypothesized that epithelial cell lines Calu-3 and A549 would produce more TNF-α, IL-6 and IL-8 mRNA following exposure to PDE than LPS alone.

B.2 Materials and methods

B.2.1 Poultry dust extract (PDE) preparation

Settled airborne dust was provided by a cage-housed poultry facility in Saskatchewan, Canada. Two grams of settled airborne dust were added to 20mL Hanks' balance salt solution and stirred at room temperature for one hour. The solubilized dust was centrifuged at 2500 rpm for 20min at 4°C. The supernatant was removed and the pellet discarded. The supernatant underwent a second round of centrifugation at 2500 rpm for 20min at 4°C. Again, the supernatant was removed and the pellet discarded. The remaining supernatant was filter sterilized using 0.22mm pore size syringe filters. This 100% PDE was stored at -80°C and diluted to 5% in cell media for cell culture treatments. Endotoxin was measured in the PDE using the LAL assay, as described previously. Cells were treated with pure LPS at the same concentration as detected in PDE.

B.2.2 Cell culture

Calu-3 cells are human airway epithelial cells and are grown in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum plus 1% penicillin/streptomycin plus 4mM L-glutamine. A549 cells are human alveolar epithelial cells and are cultivated in F-12K Medium supplemented with 10% fetal bovine serum plus 1% penicillin/streptomycin. Cells were treated with either 5% PDE or pure LPS of the same concentration for 3h. Cells were harvested and total RNA was extracted using the RNeasy MiniKit (Qiagen, Ontario, Canada), as per manufacturer's instructions.

B.2.3 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

cDNA was synthesized using QuantiTect Reverse Transcriptase (Qiagen), as per manufacturer's instructions. qRT-PCR was performed using the QuantiFast SYBR Green kit (Qiagen), as per manufacturer's instructions. Expression of the human IL-6, IL-8 and TNF-α genes was measured using primers listed in Table I. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference housekeeping gene. The PCR program was as follows: 95°C for 15min, 45 cycles of denaturation at 95°C for 1min, annealing at 55°C for 30s, and elongation at 72°C for 30s. Relative expression levels were calculated after correction for GAPDH expression.

Table I. Primers

Target gene	**Sequence**	**Hybridization temperature**	**Reference**
IL-6	GGTACATCCTCGACGGCATCT GTGCCTCTTTGCTGCTTTCAC	60	Starkie et al. 2001
IL-8	ATG ACT TCC AAG CTG GCC ACA ATA ATT TCT GTG TTG GCG	55	Journeay et al. 2009
TNF-α	ATG AGC ACT GAA AGC ATG GAG AGG TCC CTG GGG AAC	55	Journeay et al. 2009

B.3 Results

Calu-3 cells produced approximately 5-fold more IL-6 mRNA following treatment with LPS and approximately 4-fold more following PDE treatment vs. untreated cells (Figure 1). IL-8 gene expression was induced approximately 5-fold following LPS treatment and approximately 8-fold following treatment with PDE vs. untreated cells (Figure 1). Calu-3 cells produced approximately 42-fold more TNF-α mRNA following treatment with LPS and approximately 36-fold more following PDE treatment vs. untreated cells (Figure 1).

IL-6 gene expression was induced in A549 cells approximately 4-fold following LPS treatment and approximately 8-fold following treatment with PDE vs. untreated cells (Figure 2). A549 cells produced approximately 20-fold more IL-8 mRNA following treatment with LPS and approximately 38-fold more following PDE treatment vs. untreated cells (Figure 2). TNF-α gene expression was induced approximately 5-fold following LPS treatment and treatment with PDE vs. untreated cells (Figure 2).

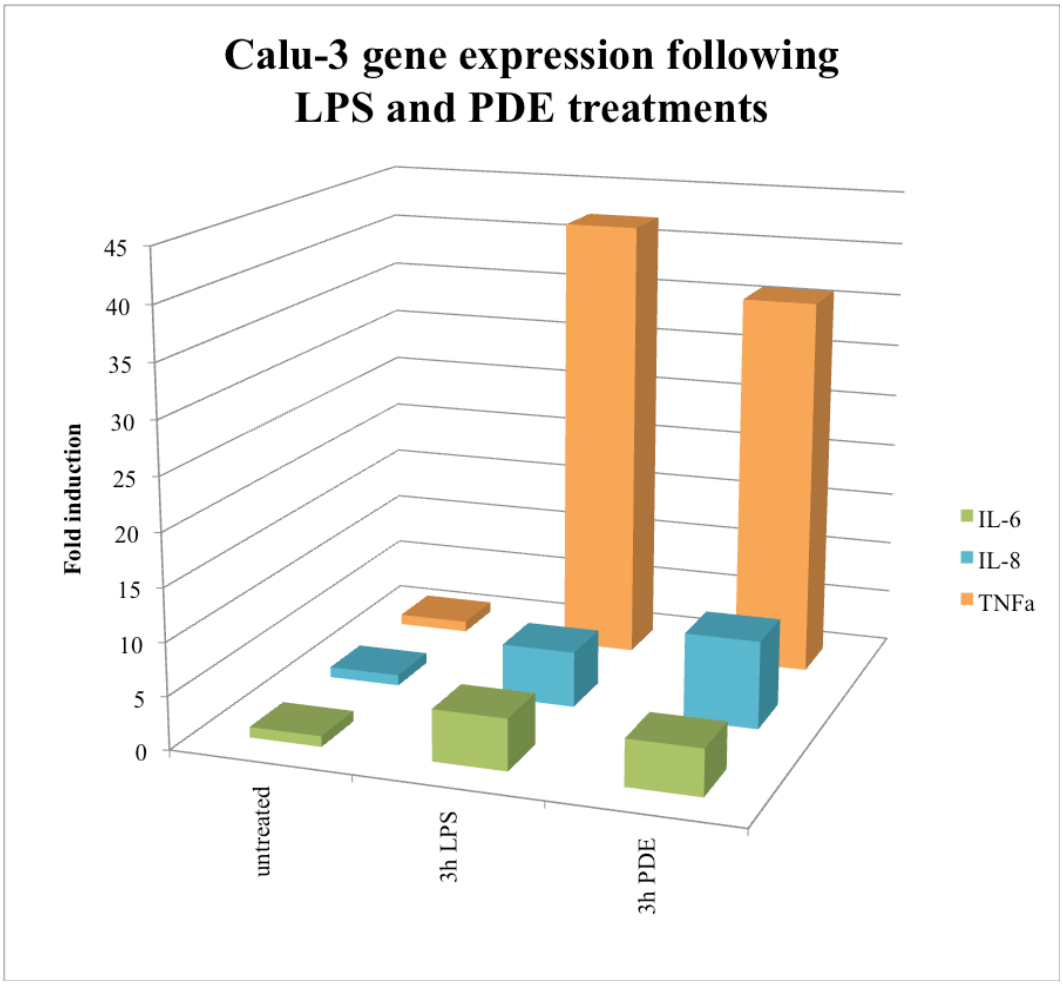

Figure 1. Calu-3 expression of IL-6, IL-8 and TNF-α genes following exposure to LPS or poultry dust extract (PDE).

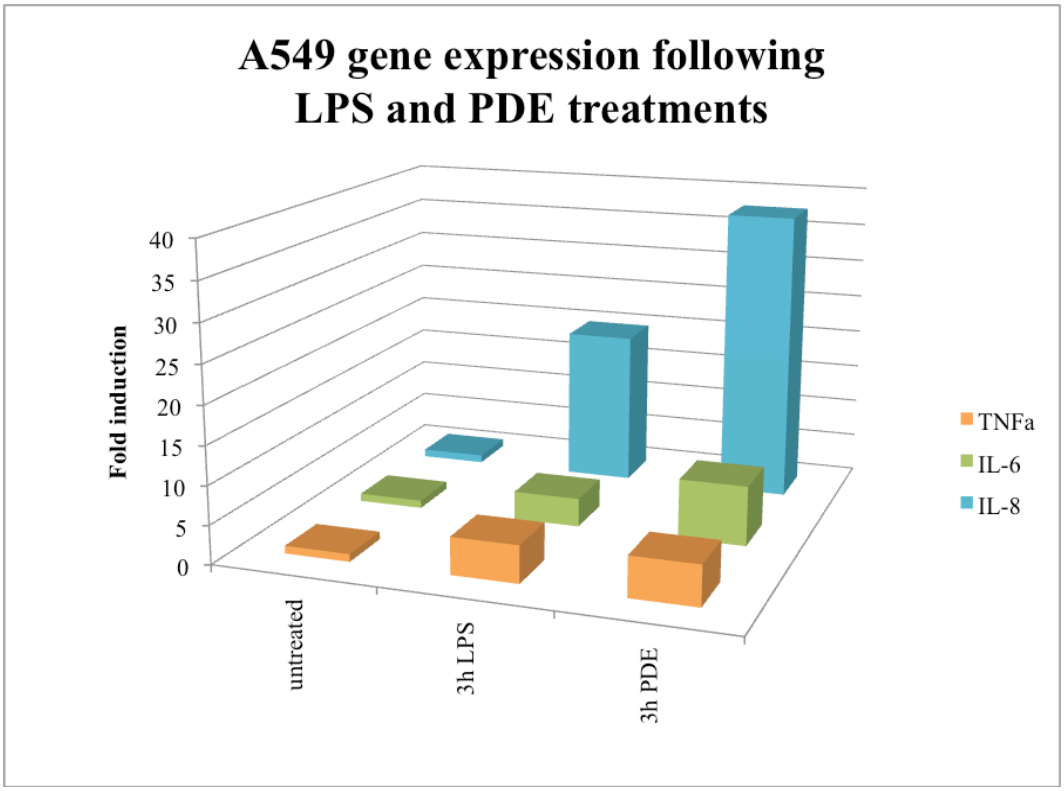

Figure 2. A549 expression of IL-6, IL-8 and TNF-α genes following exposure to LPS or poultry dust extract (PDE).

B.4 Discussion and conclusions

It was hypothesized that epithelial cell lines Calu-3 and A549 would produce more TNF-α, IL-6 and IL-8 mRNA following exposure to PDE than LPS alone. Preliminary results suggest that PDE may induce expression of some genes greater than LPS alone (Calu-3: IL-8, A549: IL-6, IL-8). However, Calu-3 cells may express more TNF-α gene following LPS treatment than treatment with PDE. Both LPS and PDE are able to induce expression of pro-inflammatory cytokine genes TNF-α, IL-6 and IL-8. Further investigation is required to determine if the responses are significantly different between the two treatments.

Calu-3 cells are bronchial epithelial cells while A549 cells are alveolar epithelial cells. In Calu-3 cells TNF-α gene expression was induced greater than that of IL-6 or IL-8. However, in A549 cells IL-8 gene expression was induced greater than expression of IL-6 or TNF-α. These data suggest that epithelial cell lines from different regions of the lung may respond differently to inflammatory stimuli (LPS or PDE). Therefore, it is important to choose the most appropriate cell line for each study and that studies using different cell lines may not be directly comparable.